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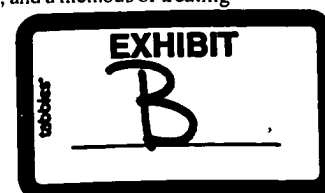
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(54) Title: RECEPTOR ANTAGONISTS FOR TREATMENT OF METASTATIC BONE CANCER

(57) Abstract: The invention provides methods of treating bone cancer, particularly metastatic bone cancer, by administering an IGF-IR antagonist and/or a PDGFR α antagonist. The invention also provides antibodies that bind to human PDGFR α and neutralize activation of the receptor. The invention further provides a methods for neutralizing activation of PDGFR α , and a methods of treating a mammal with a neoplastic disease using the antibody alone or in combination with other agents.



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RECEPTOR ANTAGONISTS FOR TREATMENT OF METASTATIC BONE CANCER

[0001] This application claims the benefit of U.S. Provisional Application No. 60/691,920.

FIELD OF THE INVENTION

[0002] The invention provides methods of treating bone cancer, particularly metastatic bone cancer, by administering an IGF-IR antagonist and/or a PDGFR α antagonist. The invention also provides antibodies that bind to human PDGFR α and neutralize activation of the receptor. The invention further provides a methods for neutralizing activation of PDGFR α , and a methods of treating a mammal with a neoplastic disease using the antibodies alone or in combination with other agents.

BACKGROUND OF THE INVENTION

[0003] Prostate cancer is the most common cancer among men, with approximately 220,000 cases and 29,000 deaths annually in the United States. A significant proportion of men diagnosed with prostate cancer have metastatic disease. Further, metastases eventually develop in many other prostate cancer patients despite treatment with surgery or radiotherapy. Bone is the most common site of prostate cancer metastasis, and is also a site to which breast cancers and lung cancers often metastasize. Most prostate cancer metastases are androgen-dependent, so that there is a rapid response to surgical or medical castration, but in virtually all patients, the tumor eventually becomes androgen-independent, leading to significant morbidity and mortality. Once bone metastases occur, currently available therapies have limited effect. The most effective approved therapy that has been described for metastatic prostate cancer (administration of docetaxel) extends median survival approximately three months. (Petrylak et al., 2004, N. Engl. J. Med. 351:1513; Tannock et al., 2004, N. Engl. J. Med. 351:1502) Accordingly, new therapies for metastatic bone cancers are urgently needed.

[0004] The insulin-like growth factor receptor (IGF-IR) is a ubiquitous transmembrane tyrosine kinase receptor that is essential for normal fetal and post-natal growth and development. IGF-IR is located on the cell surface of most cell types and serves as the signaling molecule for growth factors IGF-I and IGF-II (collectively termed henceforth IGFs). IGF-IR can stimulate cell proliferation, cell differentiation, changes in cell size, and protect cells from apoptosis. It has also been considered to be quasi-obligatory for cell

transformation (reviewed in Adams et al., *Cell. Mol. Life Sci.* 57:1050-93 (2000); Baserga, *Oncogene* 19:5574-81 (2000)). High levels of expression of IGF-IR have been reported in tissue samples from prostate cancer bone metastases. Bone contains the largest store of IGFs in the body.

[0005] IGF-IR is a pre-formed hetero-tetramer containing two alpha and two beta chains covalently linked by disulfide bonds. The receptor subunits are synthesized as part of a single polypeptide chain of 180kd, which is then proteolytically processed into alpha (130kd) and beta (95kd) subunits. The entire alpha chain is extracellular and contains the site for ligand binding. The beta chain possesses the transmembrane domain, the tyrosine kinase domain, and a C-terminal extension that is necessary for cell differentiation and transformation, but is dispensable for mitogen signaling and protection from apoptosis.

[0006] IGF-IR is highly similar to the insulin receptor (IR), particularly within the beta chain sequence (70% homology). Because of this homology, recent studies have demonstrated that these receptors can form hybrids containing one IR dimer and one IGF-IR dimer (Pandini et al., *Clin. Canc. Res.* 5:1935-19 (1999)). The formation of hybrids occurs in both normal and transformed cells and the hybrid content is dependent upon the concentration of the two homodimer receptors (IR and IGF-IR) within the cell. Although hybrid receptors are composed of IR and IGF-IR pairs, the hybrids bind selectively to IGFs, with affinity similar to that of IGF-IR, and only weakly bind insulin (Siddle and Soos, *The IGF System*. Humana Press. pp. 199-225. 1999). These hybrids therefore can bind IGFs and transduce signals in both normal and transformed cells.

[0007] A second IGF receptor, IGF-IIR, or mannose-6-phosphate (M6P) receptor, also binds IGF-II ligand with high affinity, but lacks tyrosine kinase activity (Oates et al., *Breast Cancer Res. Treat.* 47:269-81 (1998)). Because it results in the degradation of IGF-II, it is considered a sink for IGF-II, antagonizing the growth promoting effects of this ligand. Loss of the IGF-IIR in tumor cells can enhance growth potential through release of its antagonistic effect on the binding of IGF-II with the IGF-IR (Byrd et al., *J. Biol. Chem.* 274:24408-16 (1999)).

[0008] Platelet derived growth factor receptors alpha and beta (PDGFR α and PDGFR β) are type III receptor tyrosine kinases. PDGFR α is critical for development and fulfills important functions into adulthood. For example, mice homozygous for a null mutation die during embryogenesis. At later stages of development, PDGFR α is expressed in

many mesenchymal structures, whereas adjacent epithelial cells produce platelet derived growth factors (PDGFs). Tissue samples from normal or hyperplastic prostate glands test negative for PDGFR α , whereas primary prostate tumors and skeletal masses from matched subjects express PDGFR α . Further, of prostate cell lines obtained from different metastatic sites, PDGFR α is found in bone metastasis-derived PC3 cells, but not in cell lines obtained from lymph node (LNCaP) and brain (DU-145) metastases.

[0009] The platelet-derived growth factor family of growth factors consists of five different disulphide-linked dimers, PDGF-AA, -BB, -AB, -CC, and -DD, that act via PDGFR α and PDGFR β . These growth factors are dimeric molecules composed of disulfide-linked polypeptide chains that bind to two receptor proteins simultaneously and induce receptor dimerization, autophosphorylation, and intracellular signaling. PDGFR α and PDGFR β are structurally similar and can form heterodimers as well as homodimers. Because PDGFR β does not bind the PDGF-A chain with high affinity, PDGF-AA activates only $\alpha\alpha$ receptor dimers, whereas PDGF-AB and PDGF-CC activates $\alpha\alpha$ and $\alpha\beta$ receptor heterodimers.

BRIEF SUMMARY OF THE INVENTION

[0010] This invention relates to treatment of primary and metastatic bone tumors, including tumors that originate from prostate, breast, or lung and express insulin-like growth factor-I receptor (IGF-IR) and/or the alpha platelet derived growth factor receptor (PDGFR α).

[0011] The tumors to be treated can be hormone/androgen-dependent or hormone/androgen independent, and can have originated, for example, from prostate, breast, or lung.

[0012] The invention provides methods of treating a subject having a bone tumor, and methods of inhibiting growth of a bone tumor. The methods comprise administering an effective amount of an IGF-IR antagonist or an effective amount of a PDGFR α antagonist. The receptor antagonists include antibodies and antibody fragments as well as intracellular small molecule inhibitors.

[0013] The invention provides anti-IGF-IR or anti-PDGFR α antibodies that bind to their target receptor and inhibit ligand binding. The invention also provides antibodies and other antagonists that neutralize activation of IGF-IR or PDGFR α . Further certain antibodies promotes down-regulation of their target receptor, for example by internalization and/or

degradation. Accordingly, the antibodies and small molecule antagonists function to inhibit activation of downstream signaling molecules such as Akt, p42/p44, and MAPK.

[0014] The methods include use of IGF-IR or PDGFR α antagonists alone, in combination with each other or in combination with other cancer therapeutics, such as chemotherapeutics and radiation.

[0015] The invention also provides antibodies and antibody fragments that bind to PDGFR α as well as nucleotides and host cell for production of the antibodies. The antibodies block ligand binding and neutralize receptor activation. The invention also provides for use of the antibodies alone, in combination with other receptor antagonists or antineoplastic agents, or as conjugates for treatment of neoplastic disease. Anti-PDGFR α antibodies are used to treat, for example, ovarian tumors, breast tumors, lung tumors, hepatocellular tumors, gastrointestinal stromal tumors, melanomas, renal cell carcinomas, prostate tumors, and soft tissue sarcomas.

DESCRIPTION OF THE FIGURES

[0016] Figure 1 depicts growth of LuCaP 35V subcutaneous xenograft tumors in castrated SCID mice during a treatment period initiated when the tumors had reached 150-200 mm³. Panel A: untreated controls; Panel B: animals were treated for four weeks with docetaxel (either 10 mg/kg or 20 mg/kg) alone, or in combination with anti-IGF-IR antibodies (40 mg/kg IMC-A12); Panel C: serum PSA levels in untreated and treated SCID mice carrying subcutaneous LuCaP 35V xenograft tumors. Treated mice received docetaxel (20 mg/kg) alone or docetaxel (either 10 mg/kg or 20 mg/kg) in combination with anti-IGF-IR antibodies (40 mg/kg IMC-A12). Treatment was initiated when tumors had reached 150-200 mm³ and terminated after four weeks.

[0017] Figure 2 shows single cell suspensions of LuCaP 35V xenograft tumors treated with docetaxel (10 mg/kg) alone (Panel A) or in combination with anti-IGF-IR antibodies (40 mg/kg IMC-A12) (Panel B). The field labeled R1 corresponds to apoptotic cells with fragmented DNA (increased FITC labeling).

[0018] Figure 3 shows DNA synthesis (BrDu uptake) in tumor xenografts following termination of treatment with docetaxel (10 mg/kg or 20 mg/kg) alone, and in combination with anti-IGF-IR antibodies (40 mg/kg IMC-A12).

[0019] Figure 4 depicts differential expression of genes associated with prostate tumor aggressiveness (TUBB), resistance to antiandrogen therapy (BIRC 5), and apoptosis

induction (IGFBP3) in prostate tumor cells in response to treatment with docetaxel and A12 and docetaxel alone.

[0020] Figure 5 shows A12 serum levels following cessation of treatment.

[0021] Figure 6 shows body weight (a measure of overall cytotoxicity) of undiseased animals treated continuously with docetaxel (either 10 mg/kg or 20 mg/kg) alone, or in combination with anti-IGF-IR antibodies (40 mg/kg IMC-A12).

[0022] Figure 7 shows the effect of treatment with an anti-IGF-IR antibody (IMC-A12) on xenograft-produced PSA in SCID mice engrafted with LuCaP 23.1 cells.

[0023] Figure 8 shows a series of X-ray photographs of SCID mice engrafted with LuCaP 23.1 cells. A12 mice received 40 mg/ml IMC-A12 i.p. three times a week for six weeks. X-ray photographs were made at the time of sacrifice.

[0024] Figure 9 shows PSA levels (a) and representative radiographs (b) from SCID mice with intratibial xenografts of LuCaP 23.1 human prostate cells.

[0025] Figure 10 depicts the effect of human bone marrow aspirate on Akt activity in prostate cancer cells. Cell lysates were subject to SDS-PAGE. For Western blot analysis, membranes were blotted with antibodies targeting phospho-Akt (Ser-473, cell signaling Technology), PDGFR α (R&D Systems) and actin (Sigma). Primary antibody binding was detected using HRP-conjugated protein A or protein G (Sigma).

[0026] Figure 11 depicts induction and inhibition of AKT-phosphorylation in PC3-ML cells. Panel A shows the AG-1296 dose dependent inhibition of Akt phosphorylation in cells exposed to 30 ng/ml PDGF-BB. Panel B shows bone aspirate Akt phosphorylation and inhibition by 20 μ M AG-1296. Panel C show the potency of bone marrow aspirate to induce Akt phosphorylation as compared to the potency of a combination of 100 pg/ml PDGF-AA and 100 pg/ml PDGF-BB. Panel D compares the magnitudes of bone marrow aspirate induced Akt-phosphorylation, inhibition of bone marrow induced Akt-phosphorylation by AG-1296, and Akt-phosphorylation induced by PDGF-AA + PDGF-BB.

[0027] Figure 12 depicts inhibition of Akt phosphorylation in PC3-ML cells by PDGFR α antagonists. Panel A shows the dose dependent effect of monoclonal antibody IMC-3G3 on Akt phosphorylation induced by 30 ng/ml of PDGF-BB. Panels B and C provide a comparison of the effects of IMC-3G3 and AG1296 on bone marrow induced Akt phosphorylation. Panel D shows that inhibition of Akt phosphorylation is dependent on IMC-3G3 preincubation time.

[0028] Figures 13 shows binding of antibody to PDGFR α . A: direct binding of anti-PDGFR α antibody to the immobilized extracellular domain of PDGFR α . B: inhibition of [¹²⁵I]PDGF-AA binding to immobilized PDGFR α .

[0029] Figure 14 shows specific inhibition of phosphorylation of PDGFR α and downstream effector molecules.

[0030] Figure 15 shows inhibition of PDGF-AA-stimulated [³H]thymidine incorporation in PAE R α cells by mAbs.

[0031] Figure 16 shows inhibition of PDGF-AA-induced downstream-effector molecule activation in SKLMS-1 (A) and U118 (B) cells.

[0032] Figure 17 shows inhibition of PDGF-AA-stimulated [³H]thymidine incorporation in U118 (A) and SKLMS-1 (B) cells by mAbs. Inhibition of PDGF-BB-stimulated [³H]thymidine incorporation is also shown for SKLMS-1 (C) and U118 (D) cells.

[0033] Figure 18 shows dose dependent effects for treatment of established U118 (glioblastoma; panel A) and SKLMS-1 (leiomyosarcoma; panel B) tumor xenografts in nude mice.

[0034] Figure 19 shows reduction of PDGFR α phosphorylation *in vivo* in U118 tumors treated with anti-PDGFR α antibody, as compared to treatment with nonspecific human IgG.

[0035] Figure 20 depicts the GS expression vectors used for cloning hybridoma derived human V H and V κ variable regions genes and expression of complete human heavy (IgG1) and light chain proteins. The two vectors were recombined as explained in the Examples and the combined vector was transfected into NS0 cells.

DETAILED DESCRIPTION OF THE INVENTION

[0036] The present invention relates to treatment of bone tumors with antibodies or antibody fragments that bind to insulin-like growth factor-I receptor (IGF-IR). Endocrine expression of IGF-I is regulated primarily by growth hormone and produced in the liver, but other tissue types are also capable of expressing IGF-I, including bone which contains a large store of growth factors. Depending on tumor cell type, IGF-I is involved in endocrine, paracrine, and/or autocrine regulation (Yu, H. and Rohan, J., *J. Natl. Cancer Inst.* 92:1472-89 (2000)).

[0037] It has been discovered that antibodies that bind IGF-IR are useful in therapies for treatment of bone tumors that express IGF-IR. The antibodies can be use alone, or in

combination with other cancer therapeutics, particularly chemotherapeutics. Anti-IGF-IR therapy, alone or in combination with therapy with one or more anti-neoplastic agents (such as, for example, chemotherapy or radiation therapy) has significant therapeutic efficacy. Suppression of tumor growth is often accompanied with an increase in apoptosis and persists after all treatment is discontinued and tumors have again begun to grow in animals treated with chemotherapy alone.

[0038] It has also been discovered that PDGFR α plays an important role in growth of bone tumors. For example, certain tumor cell lines that express PDGFR α preferentially metastasize to bone. Such cell lines display increased PDGFR α activation and phosphorylation of downstream signaling molecules in response to soluble factors present in bone marrow. PDGFR α activation by bone marrow is reduced or completely inhibited by PDGFR α antagonists, and phosphorylation of downstream signaling molecules that are commonly activated by signaling through PDGFR α and other receptor tyrosine kinase systems is greatly reduced. Certain data suggest that the PI3K/Akt survival pathway is activated by PDGFR α signaling not only by ligands that activate PDGFR α directly, but also by factors present in bone marrow that cause transactivation of the receptor.

[0039] Primary bone tumors to be treated according to the invention include, but are not limited to, osteosarcomas, chondrosarcomas, fibrosarcomas, and hemangiosarcomas. Notably, malignant secondary (metastatic) tumors are far more common than primary bone tumors. Metastatic bone tumors to be treated according to the invention can arise from a variety of sources, the most common of which are cancers of the prostate, breast, or lung. The source of a metastatic bone cancer will usually be apparent from a patient's history. The tumors can be osteoblastic or osteolytic. The tumors may be dependent on IGF-IR stimulation when they arise, or may transition to IGF-IR dependence. For example, prostate cancers or metastases of prostate cancers that are initially hormone/androgen dependent and controllable by physical or chemical treatments that suppress androgen or hormone production, may become hormone/androgen-independent through increased sensitivity to stimulation through IGF-IR. Further, in addition to providing for treatment of hormone/androgen-independent tumors, the invention can be useful for treating hormone/androgen-dependent bone tumors without reliance on suppression of androgen or hormone production, for example, by coadministering IGF-IR antibodies with anti-neoplastic agents. Such tumors would include metastatic bone tumors that are stimulated through IGF-

IR in the IGF-rich environment of the bone, which may be sensitive to hormone stimulation but not sensitive enough to grow without IGF involvement. Hormone ablation might not be necessary for such tumors.

[0040] Bone tumors that are PDGF-dependent can also be treated according to the invention, as well as tumors that are "bone marrow" dependent. Bone marrow dependent tumors display PDGFR α activation in response to soluble factors present in bone marrow. For example, as exemplified herein, a human metastatic PDGFR α -expressing cancer cell line undergoes PDGFR α activation and Akt+ phosphorylation upon exposure to bone marrow aspirate. An anti-PDGFR α antibody and a small molecule PDGFR α antagonist each inhibit PDGFR α activation and Akt+ phosphorylation in the cell line. Soluble bone marrow factors that activate PDGFR α include, but are not limited to, PDGF-AA and -BB.

[0041] While such bone marrow dependence involves signaling through PDGFR α , it may not involve only binding of PDGFR α of a PDGFR α ligand. For example, as exemplified herein, it is noted that PDGFR α activation by defined ligands (PDGF-AA or -BB) is weaker than activation by bone marrow aspirate. Further, it is observed that in the presence of bone marrow aspirate, Akt+ phosphorylation diminishes with increased incubation time. Taken together, these results suggest that besides responding to binding of PDGFs, PDGFR α may be transactivated (phosphorylated) by other signal transduction elements (*e.g.*, other receptor tyrosine kinases) sensitive to other bone marrow components. In any event, in a cell line suited for metastatic growth in bone (*i.e.*, a cell line that preferentially metastasizes to bone), bone marrow-dependent PDGFR α activation is observed, which is inhibited by PDGFR α antagonists. Further, treatment with a PDGFR α antagonist inhibits bone marrow induced stimulation of the PI3K/Akt anti-apoptotic pathway and mitogen-activated protein kinase (MAPK).

[0042] Bone tumors to be treated with a PDGFR α antagonist can arise as metastases of prostate cancer cells, and, as above, may be hormone/androgen dependent, or have transitioned to hormone/androgen independence. Such tumors can arise as metastases of non-prostate cancers as well. One skilled in the art would easily be able to diagnose such conditions and disorders using known, conventional tests.

[0043] Treatment means any treatment of a disease in an animal and includes:
(1) preventing the disease from occurring in a mammal which may be predisposed to the disease but does not yet experience or display symptoms of the disease; *e.g.*, prevention of

the outbreak of the clinical symptoms; (2) inhibiting the disease, *e.g.*, arresting its development; or (3) relieving the disease, *e.g.*, causing regression of the symptoms of the disease. Inhibiting tumor growth includes slowing or stopping growth, as well as causing tumor regression. An effective amount for the treatment of a disease means that amount which, when administered to a mammal in need thereof, is sufficient to effect treatment, as defined above, for that disease. IGF-IR antagonists and PDGFR α antagonist of the invention may be administered alone, in combination with one another, or in combination with one or more antineoplastic agents such as, for example, a chemotherapeutic or radiologic agent.

[0044] In an embodiment of the invention, it may be desirable to determine the level of expression of IGF-IR and/or PDGFR α in a tumor to be treated. In such cases, tumor biopsies can be collected and analyzed by methods well known in the art. In another embodiment of the invention, an IGF-IR antagonist or PDGFR α antagonist is administered on the basis that the corresponding receptor is commonly expressed or activated in a particular tumor type or invariably becomes expressed or activated as the disease progresses.

[0045] An IGF-IR antagonist can be an extracellular antagonist or an intracellular antagonist and more than one antagonist may be employed. Extracellular antagonists include, but are not limited to proteins or other biological molecules that bind to IGF-IR or one or more of its ligands (*e.g.*, IGF-I and IGF-II are natural ligands of IGF-IR). In an embodiment of the invention, an extracellular antagonist inhibits binding of IGF-IR to its ligands. In one embodiment, the antagonist is an anti-IGF-IR antibody, such as, for example, IMC-A12. In another embodiment, the antagonist is a soluble ligand binding fragment of IGF-IR. Intracellular IGF-IR antagonists can be biological molecules, but are usually small molecules. Examples include, but are not limited to, tyrosine kinase inhibitor AG1024 (Calbiochem), insulin-like growth factor-I receptor kinase inhibitor NVP-AEW541 (Novartis), and insulin-like growth factor-I/insulin receptor inhibitor BMS-554417 (Bristol Myers Squibb). It will be appreciated that useful small molecule to be used in the invention are inhibitors of IGF-IR, but need not be completely specific for IGF-IR.

[0046] Anti-IGF-IR antibodies to be used according to the present invention exhibit one or more of following properties:

[0047] 1) The antibodies bind to the external domain of IGF-IR and inhibit binding of IGF-I or IGF-II to IGF-IR. Inhibition can be determined, for example, by a direct binding assay using purified or membrane bound receptor. In this embodiment, the antibodies of the

present invention, or fragments thereof, preferably bind IGF-IR at least as strongly as the natural ligands of IGF-IR (IGF-I and IGF-II).

[0048] 2) The antibodies neutralize IGF-IR. Binding of a ligand, *e.g.*, IGF-I or IGF-II, to an external, extracellular domain of IGF-IR stimulates autophosphorylation of the beta subunit and downstream signaling molecules, including MAPK, Akt, and IRS-1.

[0049] Neutralization of IGF-IR includes inhibition, diminution, inactivation and/or disruption of one or more of these activities normally associated with signal transduction. Neutralization can be determined *in vivo*, *ex vivo*, or *in vitro* using, for example, tissues, cultured cell, or purified cellular components. Neutralization includes inhibition of IGF-IR / IR heterodimers as well as IGF-IR homodimers. Thus, neutralizing IGF-IR has various effects, including inhibition, diminution, inactivation and/or disruption of growth (proliferation and differentiation), angiogenesis (blood vessel recruitment, invasion, and metastasis), and cell motility and metastasis (cell adhesion and invasiveness).

[0050] One measure of IGF-IR neutralization is inhibition of the tyrosine kinase activity of the receptor. Tyrosine kinase inhibition can be determined using well-known methods; for example, by measuring the autophosphorylation level of recombinant kinase receptor, and/or phosphorylation of natural or synthetic substrates. Thus, phosphorylation assays are useful in determining neutralizing antibodies in the context of the present invention. Phosphorylation can be detected, for example, using an antibody specific for phosphotyrosine in an ELISA assay or on a western blot. Some assays for tyrosine kinase activity are described in Panek et al., *J. Pharmacol. Exp. Thera.* 283: 1433-44 (1997) and Batley et al., *Life Sci.* 62:143-50 (1998). Antibodies of the invention cause a decrease in tyrosine phosphorylation of IGF-IR of at least about 75%, preferably at least about 85%, and more preferably at least about 90% in cells that respond to ligand.

[0051] Another measure of IGF-IR neutralization is inhibition of phosphorylation of downstream substrates of IGF-IR. Accordingly, the level of phosphorylation of MAPK, Akt, or IRS-1 can be measured. The decrease in phosphorylation is at least about 40%, and can be at least about 60%, or at least about 80%.

[0052] In addition, methods for detection of protein expression can be utilized to determine IGF-IR neutralization, wherein the proteins being measured are regulated by IGF-IR tyrosine kinase activity. These methods include immunohistochemistry (IHC) for detection of protein expression, fluorescence in situ hybridization (FISH) for detection of

gene amplification, competitive radioligand binding assays, solid matrix blotting techniques, such as Northern and Southern blots, reverse transcriptase polymerase chain reaction (RT-PCR) and ELISA. See, e.g., Grandis et al., *Cancer*, 78:1284-92 (1996); Shimizu et al., *Japan J. Cancer Res.*, 85:567-71 (1994); Sauter et al., *Am. J. Path.*, 148:1047-53 (1996); Collins, *Glia* 15:289-96 (1995); Radinsky et al., *Clin. Cancer Res.* 1:19-31 (1995); Petrides et al., *Cancer Res.* 50:3934-39 (1990); Hoffmann et al., *Anticancer Res.* 17:4419-26 (1997); Wikstrand et al., *Cancer Res.* 55:3140-48 (1995).

[0053] *Ex vivo* assays can also be utilized to determine IGF-IR neutralization. For example, receptor tyrosine kinase inhibition can be observed by mitogenic assays using cell lines stimulated with receptor ligand in the presence and absence of inhibitor. The MCF7 breast cancer line (American Type Culture Collection (ATCC), Rockville, MD) is such a cell line that expresses IGF-IR and is stimulated by IGF-I or IGF-II. Another method involves testing for inhibition of growth of IGF-IR -expressing tumor cells or cells transfected to express IGF-IR. Inhibition can also be observed using tumor models, for example, human tumor cells injected into a mouse.

[0054] The antibodies of the present invention are not limited by any particular mechanism of IGF-IR neutralization. The anti-IGF-IR antibodies of the present invention can bind externally to the IGF-IR cell surface receptor, block binding of ligand (e.g., IGF-I or IGF-II) and subsequent signal transduction mediated via the receptor-associated tyrosine kinase, and prevent phosphorylation of the IGF-IR and other downstream proteins in the signal transduction cascade.

[0055] 3) The antibodies down modulate IGF-IR. The amount of IGF-IR present on the surface of a cell depends on receptor protein production, internalization, and degradation. The amount of IGF-IR present on the surface of a cell can be measured indirectly, by detecting internalization of the receptor or a molecule bound to the receptor. For example, receptor internalization can be measured by contacting cells that express IGF-IR with a labeled antibody. Membrane-bound antibody is then stripped, collected and counted. Internalized antibody is determined by lysing the cells and detecting label in the lysates.

[0056] Another way is to directly measure the amount of the receptor present on the cell following treatment with an anti-IGF-IR antibody or other substance, for example, by fluorescence-activated cell-sorting analysis of cells stained for surface expression of IGF-IR. Stained cells are incubated at 37°C and fluorescence intensity measured over time. As a

control, part of the stained population can be incubated at 4°C (conditions under which receptor internalization is halted).

[0057] Cell surface IGF-IR can be detected and measured using a different antibody that is specific for IGF-IR and that does not block or compete with binding of the antibody being tested. (Burtrum, et al. *Cancer Res.* 63:8912-21 (2003)) Treatment of an IGF-IR expressing cell with an antibody of the invention results in reduction of cell surface IGF-IR. In a preferred embodiment, the reduction is at least about 70%, more preferably at least about 80%, and even more preferably at least about 90% in response to treatment with an antibody of the invention. A significant decrease can be observed in as little as four hours.

[0058] Another measure of down-modulation is reduction of the total receptor protein present in a cell, and reflects degradation of internal receptors. Accordingly, treatment of cells (particularly cancer cells) with antibodies of the invention results in a reduction in total cellular IGF-IR. In a preferred embodiment, the reduction is at least about 70%, more preferably at least about 80%, and even more preferably at least about 90%.

[0059] For treatment of human subjects, antibodies according to the invention are preferably human. Alternatively, the antibodies can be from non-human primates or other mammals, or be humanized or chimeric antibodies. In an embodiment of the invention, an anti-IGF-IR antibody comprises one, two, three, four, five, and/or six complementarity determining regions (CDRs) selected from the group consisting of SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:45, SEQ ID NO:47, and SEQ ID NO:49 (CDR1H, CDR2H, CDR3H, CDR1L, CDR2L, CDR3L, respectively). In another embodiment, the anti-IGF-IR antibody comprises one, two, three, four, five, and/or six complementarity determining regions (CDRs) selected from the group consisting of SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO: 55, SEQ ID NO:57, and SEQ ID NO:59 (CDR1H, CDR2H, CDR3H, CDR1L, CDR2L, CDR3L, respectively). Preferably, the antibodies (or fragments thereof) of the present invention have heavy chain CDRs of SEQ ID NO:35, SEQ ID NO:37 and SEQ ID NO:39. Alternatively and also preferably, the present antibodies including fragments thereof, have light chain CDRs of SEQ ID NO:45, SEQ ID NO:47 and SEQ ID NO:49 or SEQ ID NO:55, SEQ ID NO:57 and SEQ ID NO:59. One such anti-IGF-IR antibody is the human IgG1 antibody IMC-A12 (WO2005016970), having a heavy chain variable domain represented by SEQ ID NO:41 and a light chain variable domain represented by SEQ ID NO:51. Another preferred human antibody is IMC-2F8 (WO2005016970),

having a heavy chain variable domain identical to IMC-A12 and a light chain variable domain represented by SEQ ID NO:61. Useful antibodies further include anti-IGF-IR antibodies that compete with IMC-A12 or IMC-2F8 for binding to IGF-IR, as well as antibodies that bind to other epitopes (*i.e.*, antibodies that bind to other epitopes and exhibit properties as previously described such as ligand blocking, receptor internalization, etc., but do not compete with IMC-A12 or IMC-2F8).

[0060] According to the invention, PDGFR α antagonists can also be used for treatment. A PDGFR α antagonist can be an extracellular antagonist or an intracellular antagonist and more than one antagonist may be employed. Extracellular antagonists include, but are not limited to proteins or other biological molecules that bind to PDGFR α or one or more of its ligands (*e.g.*, PDGF-AA, -AB, -BB, -CC). In an embodiment of the invention, an extracellular antagonist inhibits binding of PDGFR α to its ligands. In one embodiment, the antagonist is an anti-PDGFR α antibody, such as, for example, IMC-3G3. In another embodiment, the binding protein is a soluble ligand binding fragment of PDGFR α . Intracellular IGF-IR antagonists can be biological molecules, but are usually small molecules. In one embodiment, the intracellular PDGFR α antagonist is AG1296. AG1296 (Calbiochem) is an inhibitor of PDGF α , PDGF β s, and c-KIT, and also reacts with Flt3. Other small molecules that target PDGFRs include STI-571 (imatinib mesylate, Gleevec®, Novartis) and SU11248 (sunitinib malate, SUTENT®, Pfizer).

[0061] In an embodiment of the invention, an anti-PDGFR α antibody comprises one, two, three, four, five, and/or six complementarity determining regions (CDRs) selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14 (CDR1H, CDR2H, CDR3H, CDR1L, CDR2L, CDR3L, respectively). Preferably, the antibodies (or fragments thereof) of the present invention have CDRs of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6. Alternatively and also preferably, the present antibodies, or fragments thereof, have CDRs of SEQ ID NO:10, SEQ ID NO:12 and SEQ ID NO:14. The amino acid sequences of the CDRs are set forth below in Table 1.

Table 1 –CDRs of IMC-3G3

Heavy Chain

CDR1	SSSYY	SEQ ID NO:2
CDR2	SFFYTGSTYYNPSLRS	SEQ ID NO:4
CDR3	QSTYYYGSGNYYGWFDK	SEQ ID NO:6

Light Chain

CDR1	RASQSVSSYLA	SEQ ID NO:10
CDR2	DASNRAT	SEQ ID NO:12
CDR3	QQRSNWPPA	SEQ ID NO:14

[0062] In another embodiment, the anti-PDGFR α antibody, or fragment thereof, has a human heavy chain variable region of SEQ ID NO:8 and/or a human light chain variable region of SEQ ID NO:16. IMC-3G3 is such an antibody and is exemplified in the present invention.

[0063] Preferably, the antibodies, or fragments thereof, of the present invention neutralize PDGFR α . Binding of a ligand, e.g., PDGF-AA, PDGF-AB, PDGF-BB or PDGF-CC, to an extracellular domain of PDGFR α stimulates receptor dimerization, autophosphorylation, activation of the receptor's internal, cytoplasmic tyrosine kinase domain, and initiation of multiple signal transduction and transactivation pathways involved in regulation of DNA synthesis (gene activation) and cell cycle progression or division. The anti-PDGFR α antibodies typically block ligand binding and/or receptor dimerization, and inhibit one or more of autophosphorylation, activation of tyrosine kinase activity and signal transduction. The anti-PDGFR α antibodies of the present invention can be specific for the extracellular ligand binding region of PDGFR α and prevent binding of a ligand of PDGFR α . Preferably, such anti-PDGFR α antibodies, or fragments thereof, bind PDGFR α at least as strongly as the natural ligands of PDGFR α . Alternatively or additionally, the antibodies can be specific for a region of the receptor monomer that would otherwise form a receptor dimer interface. Such antibodies block dimer formation, though ligand binding to a receptor monomer might or might not be blocked.

[0064] As described above for anti-IGF-IR antibodies, receptor neutralization can be determined by a variety of *in vivo*, *in vitro*, and *ex vivo* methods. In one embodiment of the invention, the anti-PDGFR α antibodies reduce phosphorylation of PDGFR α by at least about

75%. In other embodiments, phosphorylation is reduced by at least about 85% or at least about 90%. In an embodiment of the invention, as a result of inhibition of PDGFR α signal transduction, phosphorylation or a downstream signal transduction pathway component (*e.g.*, Akt, p42/p44, etc.) is reduced by at least about 40%, at least about 60%, or at least about 80%. Receptor neutralization can be determined using defined ligands (*e.g.*, PDGF-AA, -AB, -BB, -CC), mixtures of such ligands, or preparations such as bone marrow aspirates that comprise PDGFs as well as other stimulatory growth factors.

[0065] Neutralization of PDGFR α includes inhibition, diminution, inactivation and/or disruption of one or more of these activities normally associated with signal transduction. Thus, neutralizing PDGFR α has various effects, including inhibition, diminution, inactivation and/or disruption of growth (proliferation and differentiation), angiogenesis (blood vessel recruitment, invasion, and metastasis), and cell motility and metastasis (cell adhesion and invasiveness).

[0066] *Ex vivo* assays, as described above, can also be utilized to determine PDGFR α neutralization. For example, human SKLMS-1 leiomyosarcoma cells (American Type Culture Collection (ATCC), Rockville, MD; ATCC HTB-88™) or U118 glioblastoma cells (ATCC HTB-15™) stimulated with PDGF-AA can be used to assay PDGFR α inhibition. Growth inhibition can be ascertained using PDGFR α -expressing human tumor cells injected into a SCID mouse.

[0067] The present invention is not limited by any particular mechanism of PDGFR α neutralization. The anti-PDGFR α antibodies of the present invention bind externally to the PDGFR α cell surface receptor, block binding of ligand (*e.g.*, PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC), inhibit phosphorylation of the PDGFR α , inhibit signal transduction mediated via the receptor-associated tyrosine kinase, and modulate activity of downstream signal transduction components. The receptor-antibody complex can also be internalized and degraded, resulting in cell surface receptor downregulation. Matrix metalloproteinases, which function in tumor cell invasion and metastasis, can also be downregulated by the antibodies of the present invention. Moreover, antibodies of the present invention may exhibit inhibition of growth factor production and angiogenesis.

[0068] As described above, PDGFR α antagonists of the invention are useful for treating bone tumors, including metastatic bone tumors. Other tumor types that express PDGFR α and can be treated according to the invention include, but are not limited to, ovarian

tumors, breast tumors, lung tumors, hepatocellular tumors, gastrointestinal stromal tumors, melanoma, renal cell carcinoma, prostate tumors, and soft tissue sarcomas. Soft tissue sarcomas originate in such tissues as fat, muscles, nerves, tendons, and blood and lymph vessels. Typically, the tumor cells overexpress PDGFR α . PDGFR α expression can be determined, for example, by histochemistry or RNA analysis. For example, a scatchard analysis of binding of radiolabeled IMC-3G3 to U118 cells and SKLMS-1 tumor cells indicates the number of PDGFR α molecules on the cells to be about 500 and 2500, respectively.

[0069] PDGFR α antagonists function by inhibiting signal transduction by PDGFR α expressed on the tumor cells themselves, or by inhibiting PDGFR α expressed on surrounding stromal cells that otherwise undergo paracrine stimulation by PDGFs expressed from tumor cells. Thus, antibodies such as IMC-3G3 and other PDGFR α antagonists are useful for treating tumors characterized by autocrine and/or paracrine stimulation of PDGFR α .

[0070] Antibody fragments according to the invention can be produced by cleaving a whole antibody, or by expressing DNA that encodes the fragment. Fragments of antibodies may be prepared by methods described by Lamoyi et al., *J. Immunol. Methods*, 56: 235-243 (1983) and by Parham, *J. Immunol.* 131: 2895-2902 (1983). Such fragments may contain one or both Fab fragments or the F(ab')₂ fragment. Such fragments may also contain single-chain fragment variable region antibodies, i.e. scFv, dibodies, or other antibody fragments. Methods of producing such functional equivalents are disclosed in PCT Application WO 93/21319, European Patent Application No. EP 239400; PCT Application WO 89/09622; European Patent Application EP 338745; and European Patent Application EP 332424.

[0071] Preferred host cells for transformation of vectors and expression of the antibodies of the present invention are mammalian cells, e.g., COS-7 cells, Chinese hamster ovary (CHO) cells, and cell lines of lymphoid origin such as lymphoma, myeloma (e.g. NS0), or hybridoma cells. Other eukaryotic hosts, such as yeasts, can be alternatively used.

[0072] Where it is desired to express a gene construct in yeast, a suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7. Stinchcomb et al. *Nature*, 282: 39 (1979); Kingsman et al., *Gene*, 7: 141 (1979). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85: 12 (1977). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting

transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

[0073] The transformed host cells are cultured by methods known in the art in a liquid medium containing assimilable sources of carbon (carbohydrates such as glucose or lactose), nitrogen (amino acids, peptides, proteins or their degradation products such as peptones, ammonium salts or the like), and inorganic salts (sulfates, phosphates and/or carbonates of sodium, potassium, magnesium and calcium). The medium furthermore contains, for example, growth-promoting substances, such as trace elements, for example iron, zinc, manganese and the like.

[0074] High affinity anti-PDGFR α and anti-IGF-IR antibodies according to the present invention can be isolated from a phage display library constructed from human heavy chain and light chain variable region genes. For example, a variable domain of the invention can be obtained from a peripheral blood lymphocyte that contains a rearranged variable region gene. Alternatively, variable domain portions, such as CDR and FW regions, can be obtained from different sources and recombined. Further, portions of the variable domains (*e.g.*, FW regions) can be synthetic consensus sequences.

[0075] Antibodies and antibody fragments of the present invention can be obtained, for example, from naturally occurring antibodies, or Fab or scFv phage display libraries. It is understood that, to make a single domain antibody from an antibody comprising a V_H and a V_L domain, certain amino acid substitutions outside the CDRs can be desired to enhance binding, expression or solubility. For example, it can be desirable to modify amino acid residues that would otherwise be buried in the V_H-V_L interface.

[0076] Further, antibodies and antibody fragments of the invention can be obtained by standard hybridoma technology (Harlow & Lane, ed., *Antibodies: A Laboratory Manual*, Cold Spring Harbor, 211-213 (1998), which is incorporated by reference herein) using transgenic mice (*e.g.*, KM mice from Medarex, San Jose, Calif.) that produce human immunoglobulin gamma heavy and kappa light chains. In a preferred embodiment, a substantial portion of the human antibody producing genome is inserted into the genome of the mouse, and is rendered deficient in the production of endogenous murine antibodies. Such mice may be immunized subcutaneously (s.c.) with PDGFR α (usually in complete Freund's adjuvant) with boosts as needed. Immunization methods are well known in the art.

[0077] The protein used to identify IGF-IR binding antibodies of the invention is preferably IGF-IR and, more preferably, is the extracellular domain of IGF-IR. The protein used to identify PDGFR α binding antibodies of the invention is preferably PDGFR α and, more preferably, is the extracellular domain of PDGFR α . Such extracellular domains can be free or conjugated to other molecules.

[0078] The present invention also provides isolated polynucleotides encoding the antibodies, or fragments thereof, described previously. Details of the IMC-A12 anti-IGF-IR antibody are disclosed in WO2005016970. Table 2 sets forth the nucleic acid sequences for IMC-3G3.

Table 2 – Nucleotide sequences encoding CDRs of IMC-3G3

Heavy Chain

CDR1	agtagtagtt actac	SEQ ID NO:1
CDR2	agtttctttt atactgggag cacctactac aaccggtccc tcaggagt	SEQ ID NO:3
CDR3	cagtcacgt attactatgg ttggggaat tattatggct gggtcgaccg c	SEQ IS NO:5

Light Chain

CDR1	agggccagtc agagtgttag cagctactta gcc	SEQ ID NO:9
CDR2	gatgcatcca acagggccac t	SEQ ID NO:11
CDR3	cagcagcgta gcaactggcc tccggcg	SEQ ID NO:13

[0079] DNA encoding human antibodies can be prepared by recombining DNA encoding human constant regions and variable regions, other than the CDRs, derived substantially or exclusively from the corresponding human antibody regions and DNA encoding CDRs derived from a human (SEQ ID NOS:1, 3, and 5 for the heavy chain variable domain CDRs and SEQ ID NOS:9, 11, and 13 for the light chain variable domain CDRs).

[0080] Suitable sources of DNAs that encode fragments of antibodies include any cell, such as hybridomas and spleen cells, that express the full-length antibody. The fragments may be used by themselves as antibody equivalents, or may be recombined into equivalents, as described above. The DNA deletions and recombinations described in this section may be carried out by known methods, such as those described in the publications listed above with regard to equivalents of antibodies and/or other standard recombinant DNA

techniques, such as those described below. Another source of DNAs are single chain antibodies produced from a phage display library, as is known in the art.

[0081] Additionally, the present invention provides expression vectors containing the polynucleotide sequences previously described operably linked to an expression sequence, a promoter and an enhancer sequence. A variety of expression vectors for the efficient synthesis of antibody polypeptide in prokaryotic, such as bacteria and eukaryotic systems, including but not limited to yeast and mammalian cell culture systems have been developed. The vectors of the present invention can comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences.

[0082] Any suitable expression vector can be used. For example, prokaryotic cloning vectors include plasmids from *E. coli*, such as *colE1*, *pCR1*, *pBR322*, *pMB9*, *pUC*, *pKSM*, and *RP4*. Prokaryotic vectors also include derivatives of phage DNA such as *M13* and other filamentous single-stranded DNA phages. An example of a vector useful in yeast is the 2μ plasmid. Suitable vectors for expression in mammalian cells include well-known derivatives of SV40, adenovirus, retrovirus-derived DNA sequences and shuttle vectors derived from combination of functional mammalian vectors, such as those described above, and functional plasmids and phage DNA.

[0083] Additional eukaryotic expression vectors are known in the art (e.g., P.J. Southern and P. Berg, *J. Mol. Appl. Genet.*, 1, 327-341 (1982); Subramani et al., *Mol. Cell. Biol.*, 1: 854-864 (1981); Kaufmann and Sharp, "Amplification And Expression of Sequences Cotransfected with a Modular Dihydrofolate Reductase Complementary DNA Gene," *J. Mol. Biol.* 159, 601-621 (1982); Kaufmann and Sharp, *Mol. Cell. Biol.* 159, 601-664 (1982); Scahill et al., "Expression And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," *Proc. Nat'l Acad. Sci. USA* 80, 4654-4659 (1983); Urlaub and Chasin, *Proc. Nat'l Acad. Sci. USA* 77, 4216-4220, (1980).

[0084] The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the *lac* system, the *trp* system, the *tac* system, the *trc* system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast

acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters or SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

[0085] The present invention also provides recombinant host cells containing the expression vectors previously described. Antibodies of the present invention can be expressed in cell lines other than in hybridomas. Nucleic acids, which comprise a sequence encoding a polypeptide according to the invention, can be used for transformation of a suitable mammalian host cell.

[0086] Cell lines of particular preference are selected based on high level of expression, constitutive expression of protein of interest and minimal contamination from host proteins. Mammalian cell lines available as hosts for expression are well known in the art and include many immortalized cell lines, such as but not limited to, NS0 cells, Chinese Hamster Ovary (CHO) cells, Baby Hamster Kidney (BHK) cells and many others. Suitable additional eukaryotic cells include yeast and other fungi. Useful prokaryotic hosts include, for example, *E. coli*, such as *E. coli* SG-936, *E. coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DH1, and *E. coli* MRC1, *Pseudomonas*, *Bacillus*, such as *Bacillus subtilis*, and *Streptomyces*.

[0087] These present recombinant host cells can be used to produce an antibody, or fragment thereof, by culturing the cells under conditions permitting expression of the antibody or fragment thereof and purifying the antibody or fragment thereof from the host cell or medium surrounding the host cell. Targeting of the expressed antibody or fragment for secretion in the recombinant host cells can be facilitated by inserting a signal or secretory leader peptide-encoding sequence (see, Shokri et al., Appl Microbiol Biotechnol. 60(6):654-64 (2003), Nielsen et al., Prot. Eng. 10:1-6 (1997) and von Heinje et al., Nucl. Acids Res. 14:4683-4690 (1986)) at the 5' end of the antibody-encoding gene of interest. These secretory leader peptide elements can be derived from either prokaryotic or eukaryotic sequences. Accordingly suitably, secretory leader peptides are used, being amino acids joined to the N-terminal end of a polypeptide to direct movement of the polypeptide out of the host cell cytosol and secretion into the medium.

[0088] The antibodies of this invention can be fused to additional amino acid residues. Such amino acid residues can be a peptide tag, perhaps to facilitate isolation. Other

amino acid residues for homing of the antibodies to specific organs or tissues are also contemplated.

[0089] In another embodiment, an antibody of the present invention is made by expressing a nucleic acid encoding the antibody in a transgenic animal, such that the antibody is expressed and can be recovered. For example, the antibody can be expressed in a tissue specific manner that facilitates recovery and purification. In one such embodiment, an antibody of the invention is expressed in the mammary gland for secretion during lactation. Transgenic animals, include but are not limited to mice, goat, and rabbit.

[0090] Antibodies that can be used according to the invention include complete immunoglobulins, antigen binding fragments of immunoglobulins, as well as antigen binding proteins that comprise antigen binding domains of immunoglobulins. Antigen binding fragments of immunoglobulins include, for example, Fab, Fab', and F(ab')₂. Other antibody formats have been developed which retain binding specificity, but have other characteristics that may be desirable, including for example, bispecificity, multivalence (more than two binding sites), compact size (e.g., binding domains alone).

[0091] Single chain antibodies lack some or all of the constant domains of the whole antibodies from which they are derived. Therefore, they can overcome some of the problems associated with the use of whole antibodies. For example, single-chain antibodies tend to be free of certain undesired interactions between heavy-chain constant regions and other biological molecules. Additionally, single-chain antibodies are considerably smaller than whole antibodies and can have greater permeability than whole antibodies, allowing single-chain antibodies to localize and bind to target antigen-binding sites more efficiently. Furthermore, the relatively small size of single-chain antibodies makes them less likely to provoke an unwanted immune response in a recipient than whole antibodies.

[0092] Multiple single chain antibodies, each single chain having one V_H and one V_L domain covalently linked by a first peptide linker, can be covalently linked by at least one or more peptide linker to form a multivalent single chain antibodies, which can be monospecific or multispecific. Each chain of a multivalent single chain antibody includes a variable light chain fragment and a variable heavy chain fragment, and is linked by a peptide linker to at least one other chain. The peptide linker is composed of at least fifteen amino acid residues. The maximum number of amino acid residues is about one hundred.

[0093] Two single chain antibodies can be combined to form a diabody, also known as a bivalent dimer. Diabodies have two chains and two binding sites, and can be monospecific or bispecific. Each chain of the diabody includes a V_H domain connected to a V_L domain. The domains are connected with linkers that are short enough to prevent pairing between domains on the same chain, thus driving the pairing between complementary domains on different chains to recreate the two antigen-binding sites.

[0094] Three single chain antibodies can be combined to form triabodies, also known as trivalent trimers. Triabodies are constructed with the amino acid terminus of a V_L or V_H domain directly fused to the carboxyl terminus of a V_L or V_H domain, i.e., without any linker sequence. The triabody has three Fv heads with the polypeptides arranged in a cyclic, head-to-tail fashion. A possible conformation of the triabody is planar with the three binding sites located in a plane at an angle of 120 degrees from one another. Triabodies can be monospecific, bispecific or trispecific.

[0095] Thus, antibodies of the invention and fragments thereof include, but are not limited to, naturally occurring antibodies, bivalent fragments such as (Fab')₂, monovalent fragments such as Fab, single chain antibodies, single chain Fv (scFv), single domain antibodies, multivalent single chain antibodies, diabodies, triabodies, and the like that bind specifically with antigens.

[0096] The anti-IGF-IR and anti-PDGFR α antibodies or antibody fragments, which may be internalized upon binding to cells bearing IGF-IR (WO2005016970) or PDGFR α , can be chemically or biosynthetically linked to anti-tumor agents. Anti-tumor agents linked to such an antibody include any agents which destroy or damage a tumor to which the antibody has bound or in the environment of the cell to which the antibody has bound. For example, an anti-tumor agent is a toxic agent such as a chemotherapeutic agent or a radioisotope. Suitable chemotherapeutic agents are known to those skilled in the art and include anthracyclines (e.g. daunomycin and doxorubicin), methotrexate, vindesine, neocarzinostatin, cis-platinum, chlorambucil, cytosine arabinoside, 5-fluorouridine, melphalan, ricin and calicheamicin. The chemotherapeutic agents are conjugated to the antibody using conventional methods (See, e.g., Hermentin and Seiler, *Behring Inst. Mitt.* 82:197-215(1988)).

[0097] Suitable radioisotopes for use as anti-tumor agents are also known to those skilled in the art. For example, ¹³¹I or ²¹¹At is used. These isotopes are attached to the

antibody using conventional techniques (See, e.g., Pedley et al., *Br. J. Cancer* 68, 69-73(1993)).

[0098] Alternatively, the anti-tumor agent which is attached to the antibody is an enzyme which activates a prodrug. In this way, a prodrug is administered which remains in its inactive form until it reaches the target site where it is converted to its cytotoxin form. In practice, the antibody-enzyme conjugate is administered to the patient and allowed to localize in the region of the tissue to be treated. The prodrug is then administered to the patient so that conversion to the cytotoxic drug occurs in the region of the tissue to be treated.

[0099] Other anti-tumor agents include cytokines such as interleukin-2 (IL-2), interleukin-4 (IL-4) or tumor necrosis factor alpha (TNF- α). The antibody targets the cytokine to the tumor so that the cytokine mediates damage to or destruction of the tumor without affecting other tissues. The cytokine can be conjugated to the antibody at the DNA level using conventional recombinant DNA techniques.

[0100] In certain embodiments of the invention, anti-IGF-IR or anti-PDGFR α antibodies are administered in combination with one or more anti-neoplastic agents. For examples of combination therapies, see, e.g., U.S. Patent No. 6,217,866 (Schlessinger et al.) (Anti-EGFR antibodies in combination with anti-neoplastic agents); WO 99/60023 (Waksal et al.) (Anti-EGFR antibodies in combination with radiation). Any suitable anti-neoplastic agent can be used, such as a chemotherapeutic agent, radiation or combinations thereof. The anti-neoplastic agent can be an alkylating agent or an anti-metabolite. Examples of alkylating agents include, but are not limited to, cisplatin, cyclophosphamide, melphalan, and dacarbazine. Examples of anti-metabolites include, but not limited to, doxorubicin, daunorubicin, and paclitaxel, gemcitabine.

[0101] Useful anti-neoplastic agents also include mitotic inhibitors, such as taxanes docetaxel and paclitaxil. Topoisomerase inhibitors are another class of anti-neoplastic agents that can be used in combination with antibodies of the invention. These include inhibitors of topoisomerase I or topoisomerase II. Topoisomerase I inhibitors include irinotecan (CPT-11), aminocamptothecin, camptothecin, DX-8951f, topotecan. Topoisomerase II inhibitors include etoposide (VP-16), and teniposide (VM-26). Other substances are currently being evaluated with respect to topoisomerase inhibitory activity and effectiveness as anti-neoplastic agents. In a preferred embodiment, the topoisomerase inhibitor is irinotecan (CPT-11).

[0102] In an particular embodiment of the invention, an anti-IGF-IR antibody is administered in combination with docetaxel. In another embodiment of the invention, an anti-PDGFR α antibody is administered in combination with doxorubicin.

[0103] When the anti-neoplastic agent is radiation, the source of the radiation can be either external (external beam radiation therapy – EBRT) or internal (brachytherapy – BT) to the patient being treated. The dose of anti-neoplastic agent administered depends on numerous factors, including, for example, the type of agent, the type and severity tumor being treated and the route of administration of the agent. It should be emphasized, however, that the present invention is not limited to any particular dose.

[0104] The antibody (anti-IGF-IR or anti-PDGFR α) and antibody plus anti-neoplastic agent treatments can also be used for patients who receive adjuvant hormonal therapy (*e.g.*, for breast cancer) or androgen-deprivation therapy (*e.g.*, for prostate cancer).

[0105] Anti-IGF-IR and anti-PDGFR α antagonists of the invention can be coadministered, or administered with receptor antagonists that neutralize other receptors involved in tumor growth or angiogenesis. For example in an embodiment of the invention, an anti-IGF-IR antibody and an anti-PDGFR α antibody are coadministered. In one embodiment, in which a target tumor cell expresses both IGF-IR and PDGFR α , common signal transduction elements are activated by signal transduction through each receptor. Although inhibition of one receptor will generally result in decreased activation of the common downstream components, inhibition of both receptors will decrease activation further. In another embodiment, certain cells in a tumor or surrounding tissue express significant amounts of one receptor, and other cells express significant amounts of the second receptor. Coadministration of the antagonists reduces growth of the tumor cell and paracrine stimulation of surrounding cells.

[0106] A bispecific antibody can be provided as an alternative to coadministration. A variety of bispecific antibodies exist that are designed to incorporate various desirable characteristic. For example, bispecific diabodies have minimal size. Bispecific antibodies with four antigen binding sites (two for each binding specificity) have binding avidities that are similar to those of corresponding natural antibodies. Certain bispecific antibodies incorporate Fc regions, thus retaining effector functions (*e.g.*, complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC)) of natural antibodies.

WO 01/90192 describes IgG-like tetravalent antibodies WO2006/020258 describes a tetravalent antibody that incorporates two diabodies and retains effector functions.

[0107] In another embodiment, an anti-IGF-IR antibody or an anti-PDGFR α antibody or other antagonist is used in combination with a receptor antagonist that binds specifically to an epidermal growth factor receptor (*e.g.*, EGFR, Her2/erbB2, erbB3, erbB4). Particularly preferred are antigen-binding proteins that bind to the extracellular domain of EGFR and block binding of one or more of its ligands and/or neutralize ligand-induced activation of EGFR. EGFR antagonists also include antibodies that bind to a ligand of EGFR and inhibits binding of EGFR to its ligand. Ligands for EGFR include, for example, EGF, TGF- α , amphiregulin, heparin-binding EGF (HB-EGF) and betacellulin. EGF and TGF- α are thought to be the main endogenous ligands that result in EGFR-mediated stimulation, although TGF- α has been shown to be more potent in promoting angiogenesis. EGFR antagonists also include substances that inhibit EGFR dimerization with other EGFR receptor subunits (*i.e.*, EGFR homodimers) or heterodimerization with other growth factor receptors (*e.g.*, HER2). EGFR antagonists further include biological molecules and small molecules, such as synthetic kinase inhibitors that act directly on the cytoplasmic domain of EGFR to inhibit EGFR-mediated signal transduction. Erbitux® (cetuximab) is an example of an EGFR antagonist that binds to EGFR and blocks ligand binding. One example of a small molecule EGFR antagonist is IRESSA™ (ZD1939), which is a quinoxaline derivative that functions as an ATP-mimetic to inhibit EGFR. See U.S. Patent No. 5,616,582 (Zeneca Limited); WO 96/33980 (Zeneca Limited) at p. 4; *see also*, Rowinsky *et al.*, Abstract 5 presented at the 37th Annual Meeting of ASCO, San Francisco, CA, 12-15 May 2001; Anido *et al.*, Abstract 1712 presented at the 37th Annual Meeting of ASCO, San Francisco, CA, 12-15 May 2001. Another example of a small molecule EGFR antagonist is Tarceva® (OSI-774), which is a 4-(substitutedphenylamino)quinoxaline derivative [6,7-Bis(2-methoxy-ethoxy)-quinazolin-4-yl]- (3-ethynyl-phenyl)amine hydrochloride] EGFR inhibitor. See WO 96/30347 (Pfizer Inc.) at, for example, page 2, line 12 through page 4, line 34 and page 19, lines 14-17. See also Moyer *et al.*, *Cancer Res.*, 57: 4838-48 (1997); Pollack *et al.*, *J. Pharmacol.*, 291: 739-48 (1999). Tarceva® may function by inhibiting phosphorylation of EGFR and its downstream PI3/Akt and MAP (mitogen activated protein) kinase signal transduction pathways resulting in p27-mediated cell-cycle arrest. See Hidalgo *et al.*, Abstract 281 presented at the 37th Annual Meeting of ASCO, San Francisco, CA, 12-15 May 2001.

[0108] Other small molecules are also reported to inhibit EGFR, many of which are thought to be specific to the tyrosine kinase domain of an EGFR. Some examples of such small molecule EGFR antagonists are described in WO 91/116051, WO 96/30347, WO 96/33980, WO 97/27199 (Zeneca Limited), WO 97/30034 (Zeneca Limited), WO 97/42187 (Zeneca Limited), WO 97/49688 (Pfizer Inc.), WO 98/33798 (Warner Lambert Company), WO 00/18761 (American Cyanamid Company), and WO 00/31048 (Warner Lambert Company). Examples of specific small molecule EGFR antagonists include CI-1033 (Pfizer), which is a quinoxaline (N-[4-(3-chloro-4-fluoro-phenylamino)-7-(3-morpholin-4-yl-propoxy)-quinazolin-6-yl]-acrylamide) inhibitor of tyrosine kinases, particularly EGFR and is described in WO 00/31048 at page 8, lines 22-6; PKI166 (Novartis), which is a pyrrolopyrimidine inhibitor of EGFR and is described in WO 97/27199 at pages 10-12; GW2016 (GlaxoSmithKline), which is an inhibitor of EGFR and HER2; EKB569 (Wyeth), which is reported to inhibit the growth of tumor cells that overexpress EGFR or HER2 *in vitro* and *in vivo*; AG-1478 (Tryphostin), which is a quinazoline small molecule that inhibits signaling from both EGFR and erbB-2; AG-1478 (Sugen), which is a bisubstrate inhibitor that also inhibits protein kinase CK2; PD 153035 (Parke-Davis) which is reported to inhibit EGFR kinase activity and tumor growth, induce apoptosis in cells in culture, and enhance the cytotoxicity of cytotoxic chemotherapeutic agents; SPM-924 (Schwarz Pharma), which is a tyrosine kinase inhibitor targeted for treatment of prostate cancer; CP-546,989 (OSI Pharmaceuticals), which is reportedly an inhibitor of angiogenesis for treatment of solid tumors; ADL-681, which is a EGFR kinase inhibitor targeted for treatment of cancer; PD 158780, which is a pyridopyrimidine that is reported to inhibit the tumor growth rate of A4431 xenografts in mice; CP-358,774, which is a quinazoline that is reported to inhibit autophosphorylation in HN5 xenografts in mice; ZD1839, which is a quinazoline that is reported to have antitumor activity in mouse xenograft models including vulvar, NSCLC, prostate, ovarian, and colorectal cancers; CGP 59326A, which is a pyrrolopyrimidine that is reported to inhibit growth of EGFR-positive xenografts in mice; PD 165557 (Pfizer); CGP54211 and CGP53353 (Novartis), which are dianilnophthalimides. Naturally derived EGFR tyrosine kinase inhibitors include genistein, herbimycin A, quercetin, and erbstatin.

[0109] Further small molecules reported to inhibit EGFR and that are therefore within the scope of the present invention are tricyclic compounds such as the compounds described in U.S. Patent No. 5,679,683; quinazoline derivatives such as the derivatives described in

U.S. Patent No. 5,616,582; and indole compounds such as the compounds described in U.S. Patent No. 5,196,446.

[0110] Another receptor that can be targeted along with IGF-IR or PDGFR α is a vascular endothelial growth factor receptor (VEGFR). In an embodiment of the present invention, an anti-IGF-IR antibody or anti-PDGFR α antibody is used in combination with a VEGFR antagonist. In one embodiment, an antagonist is used that binds specifically to VEGFR-1/Flt-1 receptor. In another embodiment, the VEGFR antagonist binds specifically to VEGFR-2/KDR receptor. Particularly preferred are antigen-binding proteins that bind to the extracellular domain of VEGFR-1 or VEGFR-2 and block binding by their ligands (VEGFR-2 is stimulated most strongly by VEGF; VEGFR-1 is stimulated most strongly by PlGF, but also by VEGF) and/or neutralize ligand-induced activation. For example, IMC-1121 is a human antibody that binds to and neutralizes VEGFR-2 (WO 03/075840; Zhu). Another example is MAb 6.12 that binds to soluble and cell surface-expressed VEGFR-1. ScFv 6.12 comprises the V_L and V_H domains of mouse monoclonal antibody MAb 6.12. A hybridoma cell line producing MAb 6.12 has been deposited as ATCC number PTA-3344 under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the regulations thereunder (Budapest Treaty). In another embodiment, the VEGFR antagonist binds to a VEGFR ligand and blocks activation of a VEGFR by the ligand. For example, Avastin® (bevacizumab) is an antibody that binds VEGF.

[0111] Other examples of growth factor receptors involved in tumorigenesis are nerve growth factor (NGFR), and fibroblast growth factor (FGFR).

[0112] In an additional alternative embodiment, the anti-IGF-IR and anti-PDGFR α antibodies or can be administered in combination with one or more suitable adjuvants, such as, for example, cytokines (IL-10 and IL-13, for example) or other immune stimulators, such as, but not limited to, chemokine, tumor-associated antigens, and peptides. See, e.g., Larrivée et al., *supra*. It should be appreciated, however, that administration of only an anti-IGF-IR or anti-PDGFR α antibody is sufficient to prevent, inhibit, or reduce the progression of the tumor in a therapeutically effective manner.

[0113] In a combination therapy, the anti-IGF-IR or anti-PDGFR α antibody is administered before, during, or after commencing therapy with another agent, as well as any combination thereof, i.e., before and during, before and after, during and after, or before,

during and after commencing the anti-neoplastic agent therapy. For example, the antibody can be administered between 1 and 30 days, preferably 3 and 20 days, more preferably between 5 and 12 days before commencing radiation therapy. In a preferred embodiment of the invention, chemotherapy is administered concurrently with or, more preferably, subsequent to antibody therapy.

[0114] In the present invention, any suitable method or route can be used to administer antibodies of the invention, and optionally, to co-administer anti-neoplastic agents and/or antagonists of other receptors. The anti-neoplastic agent regimens utilized according to the invention, include any regimen believed to be optimally suitable for the treatment of the patient's neoplastic condition. Different malignancies can require use of specific anti-tumor antibodies and specific anti-neoplastic agents, which will be determined on a patient to patient basis. Routes of administration include, for example, oral, intravenous, intraperitoneal, subcutaneous, or intramuscular administration. The dose of antagonist administered depends on numerous factors, including, for example, the type of antagonists, the type and severity tumor being treated and the route of administration of the antagonists. It should be emphasized, however, that the present invention is not limited to any particular method or route of administration.

[0115] One of skill in the art would understand that dosages and frequency of treatment depend on the tolerance of the individual patient and on the pharmacological and pharmacokinetic properties of blocking or inhibitory agent used. Ideally, one wishes to achieve saturable pharmacokinetics for the agent used. A loading dose for both the anti-IGF-IR and anti-PDGFR α antibodies can range, for example, from about 10 to about 1000 mg/m², preferably from about 200 to about 400 mg/m². This can be followed by several additional daily or weekly dosages ranging, for example, from about 200 to about 400 mg/m². The patient is monitored for side effects and the treatment is stopped when such side effects are severe.

[0116] One of skill in the art would also know how to monitor the progress of the treatment in order to determine an effective dose. For bone metastases from prostate cancer, one such way is to monitor PSA levels. Other ways to monitor bone metastases include bone scans and MRI.

[0117] For patients for which cancer-treatment-induced bone loss (CTIBL) is a risk or problematic (e.g., patients who receive adjuvant hormonal therapy for breast cancer or

androgen-deprivation therapy for prostate cancer), the any aforementioned treatment may be supplemented by administration of agents for prevention of CTIBL, such as bisphosphonates. Bisphosphonates include, for example, clodronate, risedronate, and zoledronic acid.

[0118] Throughout this application, various publications, reference texts, textbooks, technical manuals, patents, and patent applications have been referred to. The teachings and disclosures of these publications, patents, patent applications and other documents in their entirety are hereby incorporated by reference into this application to more fully describe the state of the art to which the present invention pertains.

[0119] It is to be understood and expected that variations in the principles of invention herein disclosed may be made by one skilled in the art and it is intended that such modifications are to be included within the scope of the present invention.

[0120] The following examples further illustrate the invention, but should not be construed to limit the scope of the invention in any way. Detailed descriptions of conventional methods, such as those employed in the construction of vectors and plasmids, and expression of antibodies and antibody fragments can be obtained from numerous publications, including Sambrook, J et al., (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press; Coligan, J. et al. (1994) *Current Protocols in Immunology*, Wiley & Sons, Incorporated; Enna, S.J. et al. (1991) *Current Protocols in Pharmacology*, Wiley & Sons, Bonifacino, J.S. et al. (1999) *Current Protocols in Cell Biology*, Wiley & Sons. All references mentioned herein are incorporated in their entirety.

EXAMPLES**[0121] Example 1**

[0122] Effects of IMC-A12 and docetaxel on tumor growth. Tumor bits (20 to 30 mm³) of androgen-independent (AI) LuCaP 35V were implanted subcutaneously (*s.c.*) into 32 six-week-old castrated SCID mice respectively as previously described (4). When the implanted tumor was observed to reach a volume of 150-200 mm³, animals were randomized into four groups for treatment studies. Group 1 animals received docetaxel treatment at a dose of 20 mg/kg. Group 2 animals received docetaxel treatment at a dose of 10 mg/kg. Group 3 animals received combined treatment of 10 mg/kg docetaxel and 40 mg/kg A12. Group 4 animals received combined treatment of 20 mg/kg docetaxel and 40 mg/kg A12. All treatments were administered intraperitoneally (*ip*). Docetaxel was administered once a week. A12 was administered three times a week. All animals were treated for four weeks and monitored for additional four weeks before euthanization. Tumors were measured twice weekly and tumor volume was estimated by the formula: volume = $L \times W^2/2$. Following our University of Washington IACUC approved animal protocol, some animals were euthanized at an earlier time when tumor reached a volume of 1000 mm³ or when animal weight loss exceeded 20% of initial body weight. Animals were weighed twice a week. Blood samples were collected from orbital sinus weekly. Serum was separated and PSA level was determined using the IMx Total PSA Assay (Abott Laboratories, Abott Park, IL). BrdU was injected into the tumors 1 h before the animals were euthanized for evaluation of *in vivo* tumor cell proliferation rate.

[0123] After euthanization, tumors were collected and halved. A portion of the tumors were fixed in 10% neutral buffer formalin (NFB) and embedded in paraffin. Five micron sections were prepared for immunohistochemistry (IHC) staining. The remaining portion of the tumors was separated into single cells mechanically by mincing and filtering through 70 μ m nylon sieves.

[0124] As shown in Fig. 1, LuCaP 35V xenograft grew aggressively in mice at an average growth rate of 362.0 ± 72.0 mm³/week without any treatment. All animals in the non-treated group had to be sacrificed within three weeks after treatment initiation in experimental groups, due to tumor volume exceeds 1000 mm³. When animals were treated with 40 μ g/kg A12 alone, tumor growth rate was reduced to 192.7 ± 35.6 mm³/week during

treatment. When docetaxel was given to the animals at a dose of 10 mg/kg, LuCaP 35V tumor growth rate was reduced to an average of $29.6 \pm 6.1 \text{ mm}^3/\text{week}$. When docetaxel was given in combination with A12 treatment, LuCaP 35V tumor growth rate was further reduced to an average of $7.9 \pm 1.0 \text{ mm}^3/\text{week}$ (Fig. 1b). The inhibition effect of docetaxel combined with A12 persisted for over four weeks after the termination of treatments. When a higher dose of docetaxel (20mg/kg) was given to the animals, regardless with or without combined A12 treatment, tumor volume did not increase during the four-week treatment period; in contrast, a tendency of reduced tumor volumes was observed. However, in the four-weeks following the treatment termination, reduction of tumor volumes was continued in the group of animals treated with docetaxel combined with A12. In contrast, tumor volumes were increasing at an average rate of $27.0 \pm 16.1 \text{ mm}^3/\text{week}$ in the group of animals treated with docetaxel alone. These results have suggested that, in a given dose of docetaxel, combined treatment with A12 can enhance the inhibitory effect of docetaxel on tumor growth during treatment or after treatment follow-ups.

[0125] PSA is a commonly used clinical parameter to assess prostate tumor growth. Serum PSA levels were measured in animals during and after the treatments. As shown in Fig. 1c, in animals treated with A12 and docetaxel or 20 mg/kg docetaxel alone, no significant change was seen in serum levels of PSA during the four-week treatment, consistent with the suppressed tumor growth. After treatment termination, serum PSA level was shown increased in animals treated with docetaxel alone and, in contrast, to be consistent or even decreased in animals treated with docetaxel in combination with A12. These data are consistent with continued post-treatment inhibition of tumor growth in animals treated with docetaxel and A12.

[0126] **Induction of apoptosis by docetaxel combined with anti-IGF-IR antibody.** The combined *in vivo* effect of docetaxel and A12 treatment on cell cycle and cell survival at the experimental end point was measured by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay and propidium (PI) staining using the Apop-Direct kit (BD BioScience) as previously described. Briefly, 1×10^6 cells from the single-cell suspension were fixed with 10% neutral buffer formalin (NBF) followed by 70% ethanol alcohol at -20°C for 30 min. After several washes, cells were permeabilized with 0.1% Triton X-100 and incubated with FITC-conjugated dUTP and terminal deoxynucleotidyl transferase enzyme (TdT) at 37°C for 1 h, followed by an incubation with PI/RNase buffer (100 $\mu\text{g}/\text{ml}$ of PI, 50

µg/ml RNase) at room temperature for 60 min. Samples were analyzed by flow cytometry using a BD FACScan. Data were analyzed with CellQuest^{PRO} software.

[0127] Four weeks after treatment termination, apoptosis was detected in a significant percentage of tumors from animals that had been treated with docetaxel (66.7% in 10 mg/kg docetaxel treated group and 77.8% in 20mg/kg docetaxel treated group) in combination with A12 (Fig. 2b and Table 1), regardless of the dosage of docetaxel being used. The average apoptotic events in these tumors occurred at a rate of $15.0 \pm 4.3\%$. No apoptosis in tumors was detected in animals that were treated with docetaxel alone. Instead, a majority (88% in 10 mg/kg docetaxel treated group and 100% in 20mg/kg docetaxel treated group) of the tumors proceeded to normal cell cycle (Fig. 2a and Table 3).

Table 3 – Tumor cell cycle and survival activities at time of sacrifice				
Treatment	Apoptosis (%)	G1 arrest (%)	G2 arrest (%)	Normal cycle (%)
None	0	0	0	100
Doc (20)	0	0	0	100
Doc (20) + A12	66.7	33.3	0	0
Doc (10)	0	0	12	88
Doc (10) + A12	77.8	0	0	12.2

[0128] To further evaluate tumor cell proliferation ability after different treatment termination, paraffin-section of stained with anti-BrDu antibody. Tumor samples were fixed in 10% NBF, embedded in paraffin, and sectioned at 5-µm onto slides. After deparaffinization and rehydration, antigens were retrieved with 0.01 M citric acid (pH 6.0) at 95°C for 2 X 5 min. Slides were allowed to cool for 30 min, followed by sequential rinsing with PBS. Endogenous peroxidase activity was quenched by an incubation with 0.3% H₂O₂ in methanol for 15 min. After blocking with 1.5% normal goat serum in PBS containing 0.05% Tween 20 (PBST) for 1 h, slides were incubated with mouse anti-BrdU antibody (1 µg/ml) for 1 h followed by sequential incubation with biotinylated goat anti-mouse IgG for 30 min, peroxidase-labeled avidin for 30 min (Santa Cruz Biotechnology) and diaminobenzidine (DAB) / hydrogen peroxide chromogen substrate (Vector Laboratories, Burlingame, CA) for 5-10 min. All incubation steps were performed at room temperature. Slides were counterstained with hematoxylin (Sigma), and mounted with permount (Fisher Scientific, Fair Lawn, New Jersey). For negative control, mouse IgG (Vector Laboratories) was used instead of the primary anti-BrdU antibody. Slides were examined under a Zeiss

Microscope and digital images were obtained. Numbers of BrdU-labeled nucleus and total nucleus were collected from 10 random views of each section. Proliferation index was calculated by the number of BrdU-positive nuclei divided by the total number of nuclei. Ten fields were counted per slide. H&E stained were performed by using hematoxylin and eosin (Richard Allen, Kalamazoo, MI).

[0129] In animals that were treated with docetaxel and A12, BrDu uptake was significantly less than those treated with the same dose of docetaxel alone (Fig. 3). These data of BrDu incorporation are consistent with the above observations of cell cycle and apoptosis, suggesting that A12 significant enhanced the cytotoxicity of docetaxel.

[0130] **Differential regulation of gene expression in tumors treated with docetaxel combined with anti-IGF-IR antibody vs. docetaxel alone.** To determine potential mechanisms for the markedly enhanced effect of docetaxel by A12, IGF-IR expression was examined in all harvested tumors by immunohistochemistry and flow cytometry analysis. There was no difference in surface IGF-IR expression among all the treatment groups or compared to the control group (data not shown). Post-treatment gene expression was examined using cDNA microarray analyses in tumors from animals that had received 20 mg/kg of docetaxel and 20 mg/kg of docetaxel combined with A12. Based on SAM analyses, 49 genes were identified as differentially expressed in tumors that received combined treatment of docetaxel and A12 compared to those received docetaxel alone, with more than 2-fold change and less than 10% false discovery rate (FDR) (data not shown). Thirteen genes were identified that are potentially involved in regulation of apoptosis or cell cycle (Table 4). All 13 genes were at least 2-fold different between the two treatments and had a FDR of less than 0.02%. Nine genes were down-regulated and four genes were up-regulated in tumors treated with docetaxel and A12, as compared to tumors treated with docetaxel alone.

Table 4 - Post-treatment differential gene expression in tumors treated with docetaxel +A12 compared to tumors treated with docetaxel alone.				
HUGO	Name	GO Function	Fold Change	FDR
Down-regulated genes				
CDC2	Cell division cycle 2	cytokinesis;mitosis;	3.0	$\leq 0.02\%$
CDC6	CDC6 cell division cycle 6 homolog	negative regulation of cell proliferation	2.2	$\leq 0.02\%$
CCNA2	Cyclin A2	regulation of CDK activity	2.1	$\leq 0.02\%$
MYBL2	V-myb myeloblastosis viral oncogene homolog (avian)-like 2	anti-apoptosis;development; regulation of cell cycle;	3.2	$\leq 0.02\%$
TUBB	Tubulin beta polypeptide	microtubule-based movement taxane resistance	2.3	$\leq 0.02\%$
K-ALPHA-1	Tubulin alpha ubiquitous	microtubule-based movement taxane resistance	2.5	$\leq 0.02\%$
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)	anti-apoptosis	2.5	$\leq 0.02\%$
CDC25B	Cell division cycle 25B	positive regulation of cell proliferation	2.0	$\leq 0.02\%$
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	cell cycle arrest;	2.5	$\leq 0.02\%$
Up-regulated genes				
TOB1	Transducer of ERBB21	negative regulation of cell proliferation	2.2	$\leq 0.02\%$
CCNG2	Cyclin G2	cell cycle checkpoint	2.1	$\leq 0.02\%$
IGFBP3	Insulin-like growth factor binding protein 3	regulation of cell growth,pro-apoptotic	2.0	$\leq 0.02\%$
BIRC3	Baculoviral IAP repeat-containing 3	anti-apoptosis;cell surface receptor linked signal transduction	2.2	$\leq 0.02\%$

[00100] For selected genes, the results were confirmed by real-time RT-PCR. A standard PCR fragment of target cDNA was purified. A series of dilutions of the standards from 10ng/ μ l to 10⁻³ pg/ μ l were used for real-time RT-PCR to generate standard curves. One μ g of total RNA from each group of pooled tumor was used for first-strand cDNA synthesis using Superscript First Strand Synthesis System (Invitrogen). Real-time RT-PCR was performed in 20 μ l of reaction mixture containing 1 μ l of first strand of cDNA, specific primers sets, and Lightcycler FastStart DNA Master Plus SYBR Green using a Roche Lightcycler following the manufacturer's protocol (Roche, Nutley, NJ). RT-PCR products were subjected to melting curve analysis using Lightcycler software v3.5. The amplicon sizes were confirmed by agarose gel electrophoresis. Each sample was assayed in duplicate. The results are shown in Fig. 4.

[0131] Of the down-regulated genes, TUBB has been shown to result in resistance to docetaxel (Tanaka et al., 2004, *Int. J. Cancer* 111, 617-26), and increased expression of BIRC 5 (survivin) has been shown to be associated with aggressive prostate cancer and resistance to antiandrogen therapy (de Angelis et al., 2004, *Int. J. Oncol.* 24, 1279-88; Zhang et al., 2005, *Oncogene* 24, 2474-82). Further, TUBB is an IGF-IR-regulated gene that is involved with IGF-IR mediated transformation (Loughran et al., 2005, *Oncogene* 24, 6185-93). Of the four up-regulated genes, IGFBP3 has been shown to inhibit IGF-ligand signaling as well as to induce apoptosis in prostate tumor cells in a ligand dependent manner (Grimberg et al., 2000, *J. Cell. Physiol.* 183, 1-9).

[0132] **Post-treatment serum levels of A12.** Serum levels of A12 were measured in animals that had received docetaxel combined with A12. Serum A12 levels declined 100-fold two weeks after treatment cessation and was detected at a very low level four weeks after treatment cessation (Fig. 5).

[0133] **Overall cytotoxicity.** Cytotoxicity of coadministration of docetaxel and IMC-A12 was examined. Although A12 has greater than 95% cross-reactivity with murine IGF-IR, no abnormal daily activity or behavior changes were observed in animals treated with combined reagents or docetaxel alone compared to control tumor-bearing animals. No significant effect on kidney cells was observed in any treatment group by both cell cycle and apoptosis assays (data not shown). No significant change in body weight was observed among the treatment groups (Fig. 6).

[0134] **Anti-IGF-IR antibody therapy for bone metastases.** The effectiveness of treatment with anti-IGF-IR antibodies on metastatic growth of prostate cancer cells in bone was evaluated using prostate cancer cells injected directly into the tibia of SCID mice. By this method, metastatic tumors are established directly without reliance on chemotaxis dependent invasion from the circulation. A variety of tumor lines are available for establishing bone metastases. These include PC-3, LuCaP35, and LnCaP cells which produce osteolytic lesions and LuCaP 23.1 cells which produce osteoblastic lesions.

[0135] LuCaP 23.1 cells, which express IGF-IR, have a take rate of ~80% in the bone environment and result in osteoblastic reactions. In preliminary experiments, LuCaP 23.1 samples exhibited a significant increase in bone volume vs. tissue volume (%BV/TV) in tumor vs. control tibiae (254-503% of control, $p=0.024$). All the LuCaP 23.1 tumors in tibiae exhibited new bony trabeculae, which were not present in the normal samples, and a high

number of tumor foci, which had replaced the normal bone marrow. In some specimens the tumor and bone growth extended outside the original bone. Increased %BV/TV of LuCaP 23.1 samples was also observed after castration; the %BV/TV of tumored tibiae was 212-354% of that of non-tumored tibiae ($p=0.024$). The results observed for the intra-tibial xenografts of LuCaP 23.1 are indicative of tumor cell-stimulated *de novo* bone formation. Further, the tumors show many similarities to human samples of osteoblastic bone metastasis, including large numbers of tumor foci and increased amounts of mineralized bone.

[0136] To evaluate the effectiveness of treatment with IMC-A12, LuCaP 23.1 xenograft tumors were engrafted in SCID mice, and serum PSA levels were measured biweekly to evaluate tumor growth. All animals were castrated two weeks prior to tibial tumor cell engraftment. Administration of IMC-A12 to test mice was begun when serum PSA levels reached 5-10 ng/ml (indicating established tumors). 40 mg/kg IMC-A12 was injected i.p. three times a week for six weeks.

[0137] Bone mineral density (BMD) of the tumored tibiae and the contralateral tibiae without tumor was measured by Dual X-ray absorptiometry (PIXImus Lunar densitometer) performed on a 2.5 mm x 2.5 mm area at the tumor cell injection site, or the corresponding site of the contralateral tibia at the time of engraftment. Biweekly assessment of lesions was made by serum PSA measurements. All animals were sacrificed when the bone lesions in the control group had recurred after castration based on serum PSA levels (LuCaP 35 >60 ng/ml, ng/ml, LuCaP 23.1 >500 ng/ml), radiographical appearance of the bone lesions or when animals became compromised. One hour prior to sacrifice animals were injected with BrdU to monitor tumor cell proliferation. Radiographs were taken prior to sacrifice (FaxitronX-ray MX-20), and BMD of both tibiae were measured at the time of sacrifice.

Table 5 – Bone mineral density (BMD)				
Treatment	A12		Control	
	Tumored Leg	Non-tumored leg	Tumored leg	Non tumored leg
Mean	0.060	0.045	0.098	0.053
P value compared to control tumor	.0057			
P value compared to non-tumored leg	.0004		.0049	

[0138] Serum PSA levels were significantly lower in IMC-A12-treated mice (Fig. 7), and the increase in BMD associated with growth of osteoblastic metastatic tumors was significantly reduced as well (Table 5). BMD measurements of the non-tumored legs indicated that IMC-A12 treatment did not cause a loss of bone density (osteoporosis). Radiographs of IMC-A12-treated and untreated mice show that tumor progression was significantly reduced or prevented in treated mice (Fig. 8).

[0139] **Combination of anti-IGF-IR antibody and docetaxel for bone metastases.** SCID mice are castrated 2 weeks prior to tibial tumor injections. Bone metastases are generated by direct injection of LuCaP 23.1 prostate cancer cells into the tibia of the mice, giving rise to osteoblastic lesions. The xenografts express IGF-IR. Serum PSA levels are measured biweekly to evaluate tumor growth. When serum PSA levels reach 5-10 ng/ml indicating established tumor, animals are randomized into four groups.

[0140] In two groups, 40 mg/kg of IMC-A12 are injected i.p. three times a week for six weeks with one group receiving IMC-A12 + docetaxel 20 mg/kg i.p. once a week for 6 weeks and a second group IMC-A12 + docetaxel 10 mg i.p. three times a week for 6 weeks. Control groups receive 10 or 20 mg docetaxel i.p. without IMC-A12.

[0141] Animals are monitored with weekly PSA measurements. After termination of treatment, animals continue to be monitored with weekly PSA measurements until tumors in the docetaxel only groups show tumor regrowth. As PSA values rise in the docetaxel only groups (albeit at a slower rate than in untreated animals), the PSA levels in the IMC-A12 + docetaxel-treated mice level off, and in some animals, start to fall. Reductions in PSA levels are observed to continue, even after termination of treatment at six weeks.

[0142] As indicated above, BMD measurements are made at the time of engraftment and at sacrifice, and radiographs are taken just prior to sacrifice. The IMC-A12 + docetaxel-treated groups show little or no increase in BMD, and radiographs show little or no sign of osteoblastic activity.

[0143] **Combination of anti-IGF-IR antibody and docetaxel for bone metastases.** LuCaP 23.1 human prostate tumor bits (20 to 30 mm³) were mechanically digested. $2-5 \times 10^5$ viable LuCaP 23.1 cells were injected into the tibiae of 6 - 8 wk old SCID mice. 21 mice randomized into three groups were used for the study. After tumor injection, serum PSA was monitored weekly. Treatment started when serum PSA level reached 5-10 ng/ml, an indication of tumor growth. Group 1 received control vehicle saline buffer. Group 2 received 20 mg/kg of docetaxel i.p once a week for 4 weeks. Group 3 received 20mg/kg of docetaxel once a week and 40mg/kg of A12 i.p. three times a week for 4 weeks. To determine whether the response to treatment was osteoblastic or osteolytic, BMD was measured by DEXA-scan and x-rays of the animals at the end point of all treatments.

[0144] Docetaxel alone or docetaxel combined with A12 significantly inhibited LuCaP 23.1 tumor growth as reflected by suppression of serum PSA levels (Fig. 9a), with no significant difference between the two treatments. However, after treatment cessation, serum PSA began to increase in animals that had been treated with docetaxel alone, indicating a re-growth of the tumor; whereas continued suppression of serum PSA levels were observed in animals that received combined treatment, indicating a prolonged period of post-treatment tumor quiescence. Serum PSA levels were shown to correlate with bone density (BMD) and radiographed tumored bone sizes (Fig. 9b). At week five, the average bone density in the control, docetaxel 20, and docetaxel 20 combined with A12 treated animals was 0.112 ± 0.01 , 0.09 ± 0.02 , and 0.05 ± 0.009 (mean \pm SEM), respectively. There was an apparent trend towards a decrease in bone density with treatment.

[0145] **Example 2**

[0146] **Bone marrow aspirate-induced Akt phosphorylation.** Bone marrow samples from normal male donors (ages 18-45) were supplied by Cambrex (Poietics™ Donor Program). Samples were centrifuged at 1,500 rpm in order to separate the soluble and cellular phases. The supernatant was filtered using 0.8 μ m and 0.22 μ m filters in succession. 50 μ l of bone marrow aspirate was administered to cells in 1 ml of medium (1:20 final dilution).

[0147] For experiments performed in the presence of serum, cells were cultured in DMEM supplemented with 10% FBS and 50 $\mu\text{g/ml}$ gentamycin for 24 hours prior to exposure to bone marrow. For experiments in the absence of serum (starved cells), cells were washed twice with PBS, the growth medium was replaced with serum-free DMEM, and the cells were incubated for 4 hours prior to exposure to bone marrow preparations. When used, AG-1296, a specific inhibitor of PDGF receptors (Rice et al., 1999, *Amer. J. Path.* 155, 213-21) was added to cultures 30 min. prior to exposure to bone marrow aspirate. IMC-3G3 antibodies were administered as described at pre-treatment times as stated below.

[0148] Bone marrow activation of Akt was detected in PC3-ML cells, which express PDGFR α , but not in DU-145 cells, which lack the receptor. In one experiment, to minimize the effect of serum components on Akt activation, cells were preincubated for 4 hours in serum free media. Addition of bone marrow extracts resulted in robust Akt phosphorylation in PC3-ML cells, but not DU-145 cells. (Fig. 10A). To evaluate the significance of the response, a second experiment was conducted with serum. Robust stimulation of Akt phosphorylation in PC3-ML cells by bone marrow aspirate was also observed in the presence of serum. (Fig. 10B). Only a small response was elicited in DU-145 cells.

[0149] **PDGFR α -mediated Akt phosphorylation.** Osteoblasts and osteoclasts, which secrete both PDGF-AA and PDGF-BB, are thought to provide these growth factors in the soluble milieu of bone marrow. To determine whether the responsiveness of PC3-ML cells to bone marrow extracts was related to signal transduction through PDGFR α , PC3-ML cells were exposed to bone marrow aspirate in the absence or presence of 20 μM AG-1296. This concentration of AG-1296 completely inhibits PDGF-BB induced Akt activation. (Fig. 11A) AG-1296 inhibited bone marrow aspirate induced Akt activation by more than 40%. (Fig. 11B and D). This indicates that PDGFR α signaling is responsible for a significant proportion of bone marrow induced Akt activation.

[0150] The direct contribution of PDGF-AA and -BB to PDGFR α signaling relative to other components of bone marrow aspirates was also evaluated. It was determined that the concentrations of PDGF-AA and -BB in bone marrow aspirates from three different donors ranged from 400 pg/ml to 2 ng/ml. Given the 20-fold dilution of the bone marrow aspirates, test cells were actually being exposed to PDGF-AA and -BB concentrations between 20 and 100 pg/ml. Accordingly, PC3-ML cells were treated with 100 pg/ml each of PDGF-AA and -BB. Akt phosphorylation was less than 10% of that obtained with bone marrow aspirates.

Fig. 3C and D). Accordingly, it appears that activation of the Akt pathway by PDGFR α signaling may involve PDGFR α ligands other than PDGF-AA and -BB and/or mechanisms other than activation of PDGFR α by direct binding of a ligand.

[0151] **Inhibition of Akt phosphorylation by an anti-PDGFR α antibody.** The neutralizing antibody IMC-3G3, which is specific for human PDGFR α was also tested for its ability to inhibit Akt phosphorylation of in PC3-ML cells. A pre-incubation time of 30 minutes and a concentration of 20 μ g/ml neutralized the stimulatory effect of 30 ng/ml of PDGF-BB. (Fig. 12A) Treatment with the antibody also resulted in about 40% inhibition of bone marrow induced Akt phosphorylation (Fig. 12B and C). It was also observed that the inhibitory effect of IMC-3G3 on Akt phosphorylation was dependent on the duration of the preincubation, with a 120-minute incubation time being significantly more effective (Fig. 12D) than the 30-minute incubation time (Fig. 12B and C). One possible explanation is that IMC-3G3 induces internalization of PDGFR α , and that its inhibitory effect is related not only to blocking of ligand binding, but also to the removal of the receptor from the plasma membrane.

[0152] **Example 3**

[0153] **Isolation of Human Anti-PDGFR α Antibodies.** Human anti-PDGFR α monoclonal antibodies were generated by a standard hybridoma technology (Harlow & Lane, ed., *Antibodies: A Laboratory Manual*, Cold Spring Harbor, 211-213 (1998), which is incorporated by reference herein) using transgenic mice (Medarex Inc., Sunnyvale, CA) that express human gamma heavy and kappa light immunoglobulin chains. Human PDGFR α extracellular domain (ECD) was purchased from R&D Systems (Minneapolis, MN). KM mice were immunized subcutaneously (s.c.) with 3×10^7 porcine aortic endothelial cells stably expressing PDGFR α (PAE Ra). After 4 weeks, mice were boosted s.c. with 50 μ g PDGFR α ECD in complete Freund's adjuvant plus 3×10^7 PAE Ra cells given i.p. Mice were boosted two more times, 3 weeks apart, with 25 μ g PDGFR α ECD in incomplete Freund's adjuvant.

[0154] Splenocytes from mice with high serum binding and blocking titers were isolated and fused with myeloma cells. Hybridoma cultures displaying blocking activity were subcloned and antibodies from these hybridomas were purified by protein G chromatography.

[0155] IgGs were evaluated for binding to PDGFR α in a direct binding assay. PDGFR α ECD in PBS was immobilized onto a 96-well plate (100 ng/well). Plates were then washed with PBST (PBS + 0.05% Tween 20) and blocked with PBSM (3% milk in PBS, 200 μ L/well) for 2 hours at 25°C. IgGs diluted in PBSM were incubated with the immobilized PDGFR α ECD for 1 hr at 25°C, and the plates were washed with PBST. A secondary antibody (goat F(ab')₂ antihuman IgG-horseradish peroxidase conjugate; BioSource International, Camarillo, CA) diluted 1:5,000 in PBSM was added for 1 hour at 25°C. After the plates were washed with PBST, a TMB peroxidase substrate (KPL, Gaithersburg, MD) was added and the reaction was stopped with 100 μ L of 1 mol/L H₂SO₄. Plates were read at A450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

[0156] PDGF blocking was evaluated using a solid-phase PDGF blocking assay (see Duan et al., 1991, J. Biol. Chem. 266:413-8, which is incorporated by reference). PDGFR α ECD was diluted in PBS and coated on 96-well microtiter plates (Immulon 2HB flat-bottomed 1 x 12 Removawell strips of irradiated protein binding polystyrene; Dynex Technologies, Chantilly, VA). Each well was coated with 60 ng PDGFR α for 3 hours at 25°C in a total volume of 100 μ L. Plates were then washed twice and blocked overnight at 4°C with 25 mmol/L HEPES (pH 7.45), 0.5% gelatin, 100 mmol/L NaCl, and 0.1% Tween 20. Plates were then warmed to 25°C for 20 minutes and washed once with binding buffer (25 mmol/L HEPES (pH 7.45), 0.3% gelatin, 100 mmol/L NaCl, 0.01% Tween 20). Fifty microliters of IgGs were added to each well and incubated at 25°C for 30 minutes. Iodinated PDGF was diluted in binding buffer and added (50 μ L of a 1 nmol/L solution) to each well. Plates were incubated for 2 hours at 25°C and then washed five times with binding buffer. Each well was counted in a gamma counter. A cell-based blocking assay was done as described in Heldin et al., 1988, EMBO J. 7, 1387-93.

[0157] The kinetics of antibody binding to PDGFR α was measured using a BIAcore 3000 instrument (BIAcore, Inc., Piscataway, NJ). PDGFR α ECD was immobilized onto a sensor chip and antibody was injected at various concentrations. Sensograms were obtained at each concentration and evaluated using the BIA Evaluation 2.0 program to determine the rate constants. The affinity constant, K_d , was calculated from the ratio of rate constants K_{off}/K_{on} .

[0158] Fig. 13 shows dose-dependent binding of the human monoclonal antibody IMC-3G3 to immobilized PDGFR α ECD in the ELISA. The antibody concentration required

for 50% maximum binding to PDGFR α ECD was 0.06 nmol/L (Table 6). The ED₅₀ is consistent with the K_d for the antibody as determined by surface plasmon resonance on a BIAcore instrument (Table 1). The monoclonal antibody also blocked [¹²⁵I]PDGF-BB binding to immobilized receptor, with an IC₅₀ of 0.43 nmol/L. The binding sites for PDGF-AA and PDGF-BB on PDGFR α are not structurally coincident. The data suggests that the epitope for 3G3 spatially overlaps both growth factor binding sites.

Table 6 – Binding characteristics of anti-PDGFR α antibody					
PDGFR α binding	PDGF blocking		Binding kinetics		
(ED ₅₀ , nmol/L)	Solid phase (IC ₅₀ , nmol/L)	Cell based (IC ₅₀ , nmol/L)	K _{on} (10 ⁵ mol/L ⁻¹ s ⁻¹)	K _{off} (10 ⁻⁴ s ⁻¹)	K _d (10 ⁻⁹ mol/L)
0.06	0.24	0.58	11.50	0.47	0.04

[0159] **Inhibition of receptor phosphorylation and activation of downstream effector molecules.** The effects on PDGF-induced intracellular signaling by IMC-3G3 was determined using PAE R α cells. Cells were seeded in six-well Falcon tissue culture plates (250,000 cells per well) and allowed to grow overnight. Wells were then rinsed and incubated in serum-free medium. After an overnight incubation to render cells quiescent, the cells were treated with antibodies for 30 minutes at 37°C followed by addition of PDGF-AA or PDGF-BB and incubation for an additional 10 minutes at 37°C. Cells were then detached and lysed in 200 μ L lysis buffer (50 mmol/L Tris-HCl (pH 8.0), 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 1 mmol/L sodium orthovanadate, and protease inhibitors (Complete Mini, Roche, Mannheim, Germany)). Cell lysates were analyzed by SDS-PAGE and Western blotting using enhanced chemiluminescence reagents and Hyperfilm (Amersham Biosciences).

[0160] The antibody was tested for the ability to inhibit ligand-induced receptor tyrosine phosphorylation. PDGF-AA and PDGF-BB increase PDGFR α tyrosine phosphorylation about 5-fold at 1 and 3 nmol/L concentrations, respectively. Higher concentrations of ligand (10 nmol/L) resulted in less phosphorylated receptor possibly due to ligand-induced degradation. The antibody inhibited PDGF-BB-induced receptor to near background levels (Fig. 14A, top row). Similar data were obtained using PDGF-AA to induce receptor phosphorylation.

[0161] PDGFs transduce mitogenic signals and exert antiapoptotic effects on receptor-expressing cells through downstream effector protein. Accordingly, the monoclonal

antibody was tested for its ability to inhibit activation of MAPKs p44/p42 and Akt (involved in cell growth and antiapoptotic pathways, respectively). The anti-PDGFR α antibody inhibited phosphorylation of both MAPKs and Akt in response to PDGF-BB (Fig. 2A) and PDGF-AA (not shown). Inhibition of PDGFR α phosphorylation was dose dependent, with 50% inhibition achieved at 0.25 nmol/L (Fig. 14B).

[0162] **Antimitogenic activity.** The anti-PDGFR α monoclonal antibody was tested for its ability to block PDGFAA-induced mitogenesis of PAE R α cells. Cells were seeded in 96-well tissue culture plates (1×10^4 cells per well) and grown overnight in 100 μ L medium per well. The wells were then rinsed with serum-free medium and cells were serum starved overnight with 75 μ L serum-free medium added to each well. IgG was added (25 μ L/well) and the plates were incubated for 30 minutes at 37°C. PDGF-AA or PDGF-BB (25 μ L/well) was then added and plates were incubated for 18 to 20 hours at 37°C. Plates were incubated for an additional 4 hours after each well received 0.25 μ Ci [3 H]thymidine (25 μ L/well). Antibody, PDGF, and [3 H]thymidine were all diluted in serum-free medium. Cells were then washed with PBS plus 1% bovine serum albumin and detached by treatment with trypsin (100 μ L/well). The cells were collected onto a filter and washed thrice with double-distilled water using a MACH III cell harvester (Tomtec, Inc., Hamden, CT). After processing the filter, DNA incorporated radioactivity was determined on a scintillation counter (Wallac Microbeta, model 1450).

[0163] When IMC-3G3 was added to serum-starved PAE R α cells, PDGF-AA induced thymidine incorporation was specifically inhibited (Fig. 15) with an EC₅₀ of 8.3 nmol/L. The antibody also inhibited the 3 nmol/L PDGF-BB-induced mitogenesis of PAE R α cells with an EC₅₀ of 1.25 nmol/L (data not shown).

[0164] **Growth inhibition of human tumor cell lines expressing PDGFR α .** Human tumor cell lines expressing PDGFR α were tested to determine the affects of the human anti-PDGFR α antibody on malignant growth in *in vitro* and *in vivo* systems. Two such tumor cell lines that express PDGFR α as determined by flow cytometry are SKLMS-1 (leiomyosarcoma) and U118 (glioblastoma). These cell lines also respond to ligand in mitogenic assays and form tumors in mice. SKLMS-1 has the potential for not only paracrine but also autocrine stimulation. SKLMS-1 was shown to express PDGF-AA protein when grown in culture using a quantitative sandwich enzyme immunoassay technique (R&D Systems).

[0165] As can be seen in Fig. 16A, IMC-3G3 inhibited the phosphorylation of both Akt and MAPKs in response to PDGF-AA stimulation of SKLMS-1 cells. The inhibition of Akt phosphorylation was 100% and that of MAPKs was about 80%. The antibody is also an effective inhibitor of phosphorylation in U118 cells (Fig. 16B). Ligand-induced mitogenesis of tumor cells was also blocked. When the anti-PDGFR α antibody was added to serum-starved U118 cells, PDGF-AA-induced thymidine incorporation was specifically inhibited (Fig. 17A) with an EC₅₀ of 3.4 nmol/L. The antibody also inhibited the PDGF-AA-induced mitogenic response of SKLMS-1 cells with an EC₅₀ of 5 nmol/L (Fig. 17B), as well as the PDGF-BB-stimulated mitogenic response (Fig. 17C). Only partial inhibition (40% at 66 nmol/L; Fig. 17D) of the PDGF-BB-stimulated mitogenic response was observed for U118 cells. This is attributed to the expression of both PDGFR α and PDGFR β in those cells (data not shown).

[0166] **Inhibition of tumor xenograft growth.** IMC-3G3 was tested *in vivo* in glioblastoma (U118) and leiomyosarcoma (SKLMS-1) subcutaneous (s.c.) xenograft models in athymic nude mice. S.c. tumor xenografts were established by injecting 10×10^6 SKLMS-1 or U118 cells mixed in Matrigel (Collaborative Research Biochemicals, Bedford, MA) into female athymic nude mice (Crl:NU/NU-nuBR, Charles River Laboratories, Wilmington, MA). Tumors were allowed to reach a mean tumor volume ($\pi / 6 \times \text{longest length} \times \text{perpendicular width}^2$) of about 400 mm³. The mice were randomized into five groups (n = 12) and treated by i.p. injection twice weekly for the duration of the study. Group 1 mice were treated with vehicle control (0.9% NaCl, USP for Irrigation, B/Braun). Groups 2 to 4 mice were treated with 6, 20, and 60 mg/kg of the instant anti-PDGFR α antibody. Group 5 mice were treated with 60 mg/kg human IgG (Sigma). Groups treated with 6, 20, or 60 mg/kg anti-PDGFR α antibody or human IgG were given 21.4, 71.4, and 214 mg/kg loading doses, respectively. The loading doses were calculated to achieve a steady state plasma concentration from the first dose (elimination half-life, 7 days) using a dosing regimen of twice weekly. Tumor volumes were evaluated twice weekly and tumor growth in the treatment groups was compared with a repeated measures ANOVA.

[0167] As shown in Fig. 18A, human IgG had no effect on glioblastoma growth compared with saline treated mice (P = 0.74), whereas the anti-PDGFR α antibody significantly inhibited tumor growth at 6 (P = 0.06), 20 (P = 0.03), and 60 (P = 0.0004) mg/kg doses. At the end of the U118 study, the %T/C [(average tumor volume for the 3G3-treated

group at conclusion of study / average tumor volume at beginning of treatment) / (average tumor volume for control-treated group at conclusion of study / average tumor volume at beginning of treatment) x 100] values were 67%, 63%, and 35% for 6, 20, and 60 mg/kg 3G3-treated dose groups, respectively. Further, tumor regression was observed in 4 of 12, 5 of 11, and 10 of 12 animals in the 6, 20, and 60 mg/kg treatment groups. There were no regressions in either control group.

[0168] Fig. 18B shows that leiomyosarcoma growth was also significantly inhibited by treatment at 6 ($P = 0.02$), 20 ($P = 0.003$), and 60 ($P < 0.0001$) mg/kg. The final %T/C values were 66%, 57%, and 31% for the 6, 20, and 60 mg/kg treatment groups, respectively with no tumor regressions.

[0169] Histologic examination of xenografts at the end of treatment showed marked differences in tumors from treated animals as compared with tumors from animals receiving control therapy. Resected tumors were fixed in QDL fixative at 4°C for 24 hours. After paraffin embedding and sectioning at 4 μ m, formalin-fixed sections were stained with Mayer's H&E (Richard Allen, Kalamazoo, MI).

[0170] In the U118 group treated with the highest dose (60 mg/kg), fewer viable tumor cells were found and there were substantially more cell-sparse regions compared with the saline-control group (Fig. 18C). Treated SKLMS-1 xenografts at day 25 also showed a reduction in the amount of viable tumor cells and cellular packing compared with the saline-control group (Fig. 18D).

[0171] ***In vitro* inhibition of PDGFR α -mediated stimulation of a glioblastoma line.** The level of receptor phosphotyrosine in U118 tumors was evaluated one week after treatment with anti-PDGFR α antibody or human IgGt. Mice with established U118 tumors (500 mm³) were treated with a 214 mg/kg loading dose followed 72 hours later by a 60 mg/kg maintenance dose of antibody. Tumors were harvested from mice one week (168 hours) after the first antibody injection (at a time before tumor regression is observed on average; see Fig. 18A) and homogenized in phosphorylation assay lysis buffer (see above). The lysates were centrifuged twice at 14,000 rpm and the protein concentration for the collected supernatant was determined (Bio-Rad protein assay, Bio-Rad, Hercules, CA). Lysate (4 mg) from each sample was immunoprecipitation using anti-PDGFR α antibody. Immunoprecipitated human PDGFR α was then immunoblotted with either an anti-PDGFR or anti-phosphotyrosine antibody. Fig. 19 shows that administration of anti-PDGFR α antibody

resulted in reduction in the level of PDGFR α phosphotyrosine relative to a human IgG control in these tumors.

[0172] **Cell line engineering.** First, the genes encoding the heavy and light chain variable domains of the human anti-PDGFR α antibody were cloned and sequenced. A primer series was obtained from MEDAREX that anneals to the 5' and 3' flanking sequences of the human immunoglobulin variable region sequences within MEDAREX-derived hybridomas. The heavy chain variable region amplified with primer pair AB88 (forward) and AB90 (reverse) (Table 7). Light chain products were amplified with primer pairs containing the forward primer AB182 and reverse primer AB16 (Table 7). The 0.4kb products of these reactions were cloned into the vector ZeroBlunt (Invitrogen) to produce AB88-1 (V_H) and AB182-3 (V_K), and the inserts were sequenced with universal T7 and M13R primers.

Table 7 – Primers for MEDAREX hybridomas			
Oligo	Size	DNA sequence (5'-3')	SEQ ID NO
AB88	21	ATGAAACACCTGTGGTTCTTC	20
AB90	21	TGCCAGGGGAAGACCGATGG	21
AB182	24	ATGGAA (G/A) CCCCAGCGCAGCTTCTC	22
AB16	20	CGGGAAGATGAAGACAGATG	23

[0173] In order to generate plasmid vectors for expressing the complete IgG1 antibody, the cloned variable regions were PCR amplified and ligated in two steps into expression vectors containing constant region genes. Primary PCR heavy chain amplification utilized 25ng of plasmid AB88-1 as template for primers IPHF5 (forward) and IPHR5 (reverse). Secondary PCR heavy chain amplification utilized 5 μ l primary reaction as template and the primers OPSIF and IPHR5. The combination of the two forward primers add a 57 base pair sequence to the 5' end of the immunoglobulin genes encoding a 19 amino acid mouse heavy chain gene signal sequence (MGWSCILFLVATATGVHS; SEQ ID NO:24) for efficient immunoglobulin processing and secretion. In addition, the forward primer OPSIF adds a consensus "Kozak" sequence (*J. Mol. Biol.* 196:947) for efficient initiation of translation of these genes in mammalian cells and a 5' HindIII restriction endonuclease site for cloning of the amplified product into the suitable expression vector. The heavy chain reverse primer contains an inframe NheI site for cloning into the constant region vector.

[0174] PCR was performed in two steps utilizing the Expand PCR kit (Boehringer Mannheim Inc.) according to manufacturer's specifications using Expand Buffer system #3 in 50 μ l reactions with the following cycling conditions:

1 cycle	94°, 2 minutes
5 cycles	94°, 20 seconds
	48°, 60 seconds
	68°, 2 minutes
20 cycles	94°, 20 seconds
	65°, 60 seconds
	68°, 2 minutes
1 cycle	68°, 5 minutes

After two rounds of PCR, the product was purified following agarose gel electrophoresis and cloned as a HindIII-NheI digested fragment into vector pDFc (Fig. 8), which contains the human gamma 1 constant region.

[0175] Primary PCR light chain amplification utilized 25ng of pAB182-3 plasmid as template primers IPLF4 (forward) and IPLR2 (reverse). Secondary PCR light chain amplification utilized 5 μ l primary reaction as template and the primers OPSIF and IPLR2. As for the heavy chain, the two forward primers provide a secretion signal sequence. The light chain reverse primer contains an in-frame BsiWI site for cloning into the kappa constant region vector pLck (Fig. 8). PCR reactions were performed as for the heavy chain above. After two rounds of PCR, the product was purified following agarose gel electrophoresis and cloned into pLck, which contains the human kappa light chain constant region.

Table 8 – Primers for V _H and V _K expression vectors			
Oligo	Size	DNA sequence (5'-3')	SEQ ID NO
OPSIF	53	GAGAAGCTTGCCGCCACCATGGGATGGTCATGTATCAT CCTTTTCTAGTAGC	25
IPHF5	58	TCCTTTTCTAGTAGCAACTGCAACTGGAGTACATTCA CAGCTGCAGCTGCAGGAGTC	26
IPHR5	37	CGCGCTAGCTGAGGAGACGGTGACCAGGGTTCCTGG	27
IPLF4	58	TCCTTTTCTAGTAGCAACTGCAACTGGAGTACATTTCAG AAATTGTGTTGACACAGTC	28
IPLR2	37	GCGCGTACGTTTGATTTCACCTTGGTCCCTTGGCCG	29

[0176] In order to generate a single plasmid vector for stable transfection, the heavy chain expression cassette, containing the CMV promoter, heavy chain coding region, and polyA element were cloned into the light chain vector as a NotI-SalI fragment (Fig. 20).

[0177] This construct was then utilized to generate a stable production line in myeloma cell line NS0 cells. NS0 cells were transfected with the expression plasmid via electroporation using the BioRad Gene Pulser II. Prior to transfection, the plasmid DNA was linearized with PvuI, ethanol precipitated, and resuspended at a concentration of 0.4mg/ml (40ug in 100ul dH₂O). Cells were electroporated with the 40ug of DNA in a final volume of 800ul by a single pulse of 250 volts, 400μFd. Electroporated cells were dispersed in 50ul aliquots in DMEM medium (JRH Biosciences Inc.) containing 10% dialysed fetal calf serum (dFCS) (Hyclone, Lot#: AHA7675) and 2mM glutamine (Invitrogen/Life Technologies) into wells of approximately eighteen 96 well plates at a density of 5,000 – 10,000 cells per well. Selection for glutamine synthetase (GS) positive transfectants was initiated 24 hours later by the addition of glutamine free DMEM containing 10% dFCS and supplemented with 1x GS supplement (JRH Biosciences Inc.). Cells were cultured for 2-4 weeks at 37°C, 5% CO₂ to enable the growth and expansion of colonies. More than 300 colonies were screened using an anti-human Fc (gamma) ELISA (Horseradish peroxidase detection at A450nm). Antibody expressing clones (58%) were expanded and retested for productivity over 3-5 days cultivation. To adapt cells into serum free medium, positive cell lines were expanded by the addition of an equal volume of serum free GS-OS cultivation medium at each passage. Strong positives, producing 25ug/ml or more in 3 day sub-confluent 24 well cultures, were expanded for further analysis to complete adaptation to serum free medium.

[0178] It is understood and expected that variations in the principles of invention herein disclosed may be made by one skilled in the art and it is intended that such modifications are to be included within the scope of the present invention.

What is claimed is:

1. A method of treating a subject having a bone tumor, which comprises administering an effective amount of an IGF-IR antagonist.
2. A method of inhibiting growth of a bone tumor, which comprises administering an effective amount of an IGF-IR antagonist.
3. A method of treating a subject having a bone tumor, which comprises administering an effective amount of a PDGFR α antagonist.
4. A method of inhibiting growth of a bone tumor, which comprises administering an effective amount of a PDGFR α antagonist.
5. The method of any one of Claims 1 to 4, wherein the bone tumor is a primary tumor.
6. The method of any one of Claims 1 to 4, wherein the bone tumor is a secondary tumor.
7. The method of any one of Claims 1 to 4, wherein the tumor cell growth is androgen-dependent.
8. The method of any one of Claims 1 to 4, wherein the tumor cell growth is androgen-independent.
9. The method of any one of Claims 1 to 4, wherein the tumor is metastasized from a prostate cancer.
10. The method of any one of Claims 1 to 4, wherein the tumor is metastasized from a breast cancer.
11. The method of any one of Claims 1 to 4, wherein the tumor is metastasized from a lung cancer.
12. The method of Claim 1 or 2, wherein the IGF-IR antagonist is an antibody or antibody fragment.

13. The method of Claim 12, wherein the antibody or antibody fragment competes for binding to IGF-IR with an antibody comprising a heavy chain variable domain having SEQ ID NO:41 and a light chain variable domain having SEQ ID NO:51.

14. The method of Claim 1 or 2, wherein the IGF-IR antagonist is an intracellular inhibitor of IGF-IR.

15. The method of Claim 14, wherein the intracellular IGF-IR inhibitor is selected from the group consisting of AG1024, NVP-AEW541, and BMS-554417.

16. The method of Claim 3 or 4, wherein the PDGFR α antagonist is an antibody or antibody fragment.

17. The method of Claim 16, wherein the antibody or antibody fragment competes for binding to PDGFR α with an antibody comprising a heavy chain variable domain having SEQ ID NO:8 and a light chain variable domain having SEQ ID NO:16.

18. The method of Claim 3 or 4, wherein the PDGFR α antagonist is an intracellular inhibitor of PDGFR α .

19. The method of Claim 18, wherein the intracellular PDGFR α inhibitor is selected from the group consisting of AG1296, STI-571 and SU11248.

20. The method of Claim 12 or 16, wherein the antibody or antibody fragment is human.

21. The method of Claim 12 or 16, wherein the antibody or antibody fragment is humanized.

22. The method of Claim 12 or 16, wherein the antibody or antibody fragment is chimeric.

23. The method of Claim 1 or 2, wherein the IGF-IR antagonist inhibits binding of IGF-I or IGF-II to IGF-IR.

24. The method of Claim 1 or 2, wherein the IGF-IR antagonist neutralizes activation of IGF-IR by IGF-I or IGF-II.

25. The method of Claim 1 or 2, wherein the IGF-IR antagonist reduces IGF-IR surface receptor concentration.

26. The method of Claim 1 or 2, wherein the IGF-IR antagonist is an antibody that binds to IGF-IR with a K_d of about $3 \times 10^{-10} \text{ M}^{-1}$ or less.
27. The method of Claim 1 or 2, wherein the IGF-IR antagonist inhibits phosphorylation of a signaling molecule downstream of IGF-IR.
28. The method of Claim 3 or 4, wherein the PDGFR α antagonist inhibits binding of a platelet derived growth factor to PDGFR α .
29. The method of Claim 3 or 4, wherein the PDGFR α antagonist neutralizes activation of PDGFR α .
30. The method of Claim 3 or 4, wherein the PDGFR α antagonist reduces PDGFR α surface receptor concentration.
31. The method of Claim 3 or 4, wherein the PDGFR α antagonist is an antibody that binds to IGF-IR with a K_d of about 10^{-9} M^{-1} or less.
32. The method of Claim 3 or 4, wherein the PDGFR α antagonist inhibits phosphorylation of a signaling molecule downstream of PDGFR α .
33. The method of Claim 3 or 4, wherein the PDGFR α antagonist inhibits bone marrow induced activation of Akt.
34. The method of any one of Claims 1 to 4, which further comprises coadministering a second receptor tyrosine kinase antagonist.
35. The method of Claim 1 or 2, wherein the IGF-IR antagonist is a bispecific antibody.
36. The method of Claim 35, wherein the bispecific antibody is specific for IGF-IR and PDGFR α .
37. The method of Claim 35, wherein the bispecific antibody is specific for IGF-IR and EGFR.
38. The method of Claim 3 or 4, wherein the PDGFR α antagonist is a bispecific antibody.

39. The method of Claim 38, wherein the bispecific antibody is specific for PDGFR α and EGFR.

40. The method of any one of Claims 1 to 4, which further comprises administering an effective amount of an anti-neoplastic agent.

41. The method of Claim 1, wherein the anti-neoplastic agent is docetaxel.

42. The method of Claim 1, wherein the anti-neoplastic agent is doxorubicin.

43. The method of Claim 1, wherein the anti-neoplastic agent is radiation.

44. An isolated human antibody or antibody fragment specific for PDGFR α comprising one or more complementarity determining regions selected from the group consisting of SEQ ID NO:2 at CDRH1; SEQ ID NO:4 at CDRH2; SEQ ID NO:6 at CDRH3; SEQ ID NO:10 at CDRL1; SEQ ID NO:12 at CDRL2; and SEQ ID NO:14 at CDRL3.

45. The antibody or antibody fragment of Claim 44, which comprises SEQ ID NO:2 at CDRH1; SEQ ID NO:4 at CDRH2; and SEQ ID NO:6 at CDRH3.

46. The antibody or antibody fragment of Claim 44, which comprises SEQ ID NO:8.

47. The antibody or antibody fragment of Claim 44, which comprises SEQ ID NO:10 at CDRL1; SEQ ID NO:12 at CDRL2; and SEQ ID NO:14 at CDRL3.

48. The antibody or antibody fragment of Claim 44, which comprises SEQ ID NO:16.

49. The antibody or antibody fragment of Claim 44, which comprises SEQ ID NO:2 at CDRH1; SEQ ID NO:4 at CDRH2; SEQ ID NO:6 at CDRH3; SEQ ID NO:10 at CDRL1; SEQ ID NO:12 at CDRL2; and SEQ ID NO:14 at CDRL3.

50. The antibody or antibody fragment of Claim 44, which comprises SEQ ID NO:8 and SEQ ID NO:16.

51. The antibody or antibody fragment of any one of Claims 44 to 50, which binds selectively to PDGFR α .

52. The antibody or antibody fragment of any one of Claims 44 to 50, which inhibits binding of PDGFR α to a ligand of PDGFR α .

53. The antibody or antibody fragment of any one of Claims 44 to 50, which neutralizes PDGFR α .

54. The antibody fragment of any one of Claims 44 to 50, which is selected from the group consisting of a single chain antibody, an Fab, a single chain Fv, a diabody, and a triabody.

55. A conjugate of the antibody or antibody fragment of any one of Claims 44 to 50.

56. The conjugate of Claim 55, which comprises an anti-neoplastic agent, a target moiety or a reporter moiety.

57. An isolated polynucleotide which encodes an antibody or antibody fragment and comprises one or more nucleotide sequences selected from the group consisting of SEQ ID NO: 1 at CDRH1; SEQ ID NO:3 at CDRH2; SEQ ID NO:5 at CDRH3; SEQ ID NO:9 at CDRL1; SEQ ID NO:11 at CDRL2; and SEQ ID NO:13 at CDRL3.

58. The isolated polynucleotide of Claim 57, which comprises SEQ ID NO:7.

59. The isolated polynucleotide of Claim 57, which comprises SEQ ID NO:15.

60. An expression vector comprising the polynucleotide of any one of Claims 57 to 59.

61. A recombinant host cell comprising the expression vector of Claim 60.

62. The recombinant host cell of Claim 61 which produces a polypeptide comprising SEQ ID NO:8 and a polypeptide comprising SEQ ID NO:16.

63. The recombinant host cell of Claim 61 which produces a polypeptide comprising SEQ ID NO:8 and SEQ ID NO:16.

64. A method of neutralizing activation of PDGFR α in a mammal comprising administering an effective amount of the antibody of any one of Claims 44 to 54.

65. A method of inhibiting tumor growth in a mammal comprising administering a therapeutically effective amount of the antibody of any one of Claims 44 to 54.

66. The method of Claim 65, wherein the tumor expresses PDGFR α .

67. The method of Claim 65, wherein the tumor overexpresses PDGFR α .

68. The method of Claim 65, wherein the tumor is a primary tumor.
69. The method of Claim 65, wherein the tumor is a metastatic tumor.
70. The method of Claim 65, wherein the tumor is a refractory tumor.
71. The method of Claim 65, wherein the tumor is a vascularized tumor.
72. The method of Claim 65, wherein the tumor is selected from the group consisting of an ovarian tumor, a breast tumor, a lung tumor, a hepatocellular tumor, a gastrointestinal stromal tumor, a melanoma, a renal cell carcinoma, a prostate tumor, and a soft tissue sarcoma.
73. The method of Claim 65, wherein the antibody or antibody fragment is administered in combination with an anti-neoplastic agent.
74. The method of Claim 73, wherein the antineoplastic agent is a chemotherapeutic agent.
75. The method of Claim 73, wherein the antineoplastic agent is doxorubicin.
76. The method of Claim 73, wherein the antineoplastic agent is radiation.
77. The method of Claim 65, wherein the antibody or antibody fragment is administered with a second PDGFR α antagonist.
78. The method of Claim 77, wherein the PDGFR α antagonist is an intracellular PDGFR α antagonist.
79. The method of Claim 65, which further comprises administration of a therapeutically effective amount of an epithelial growth factor receptor (EGFR) antagonist.
80. The method of Claim 65, which further comprises administration of a therapeutically effective amount of an insulin like growth factor receptor (IGF-IR) antagonist.

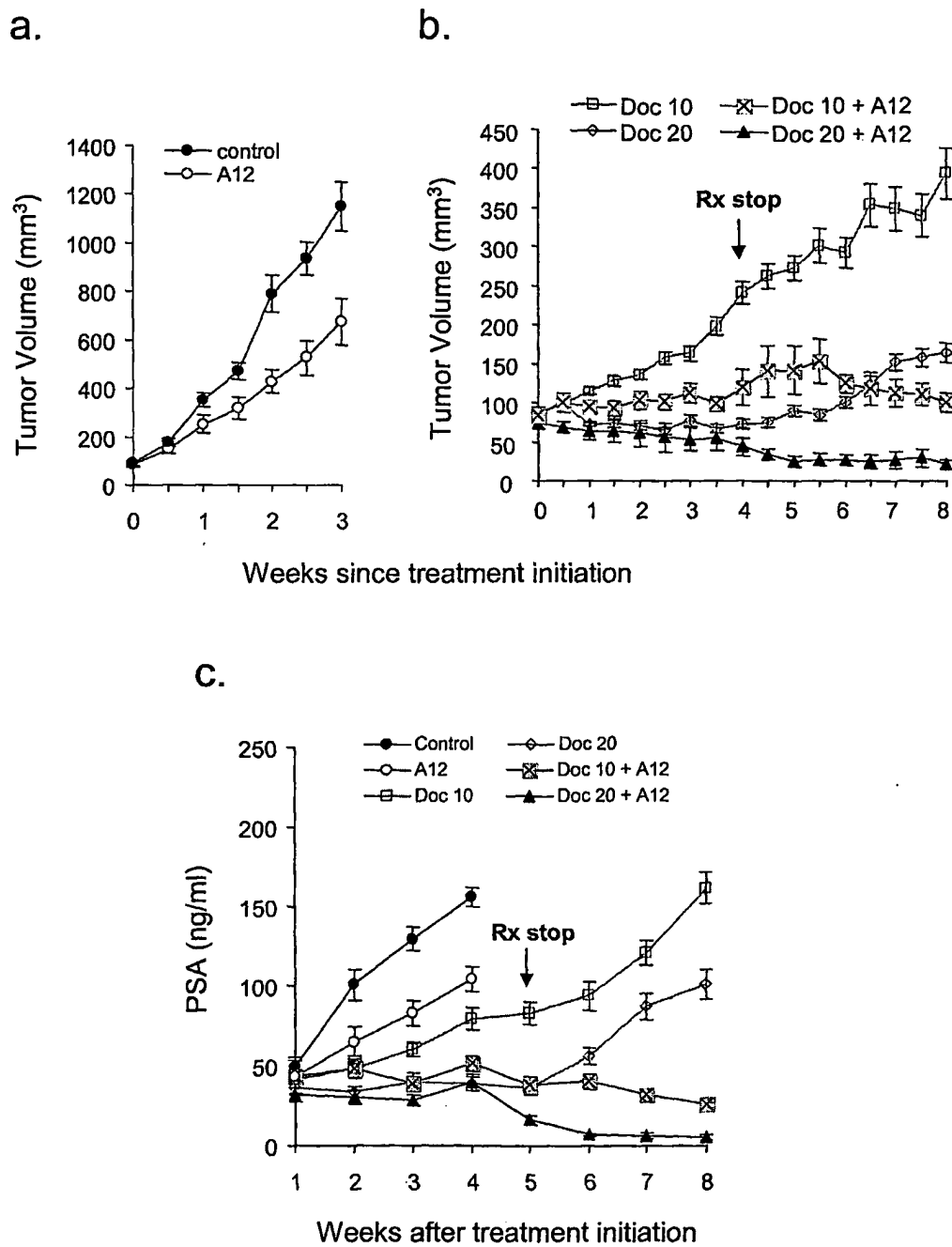


Fig. 1

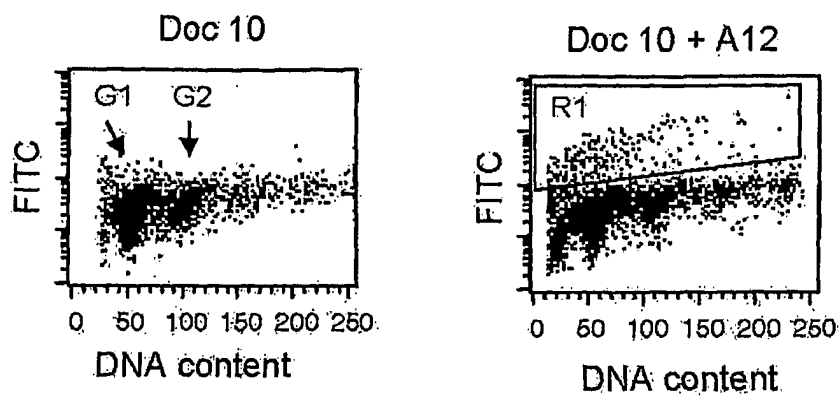


Fig. 2

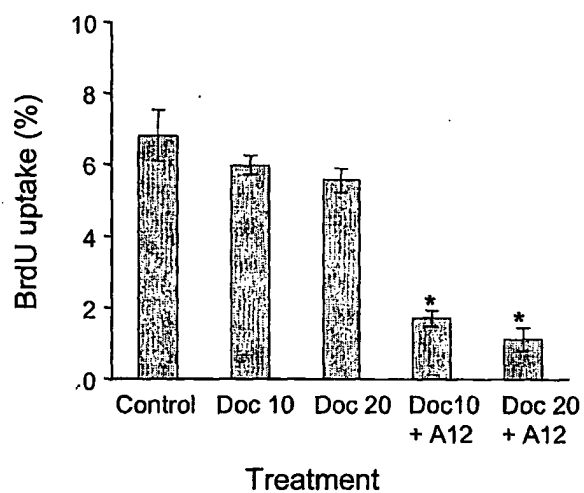


Fig. 3

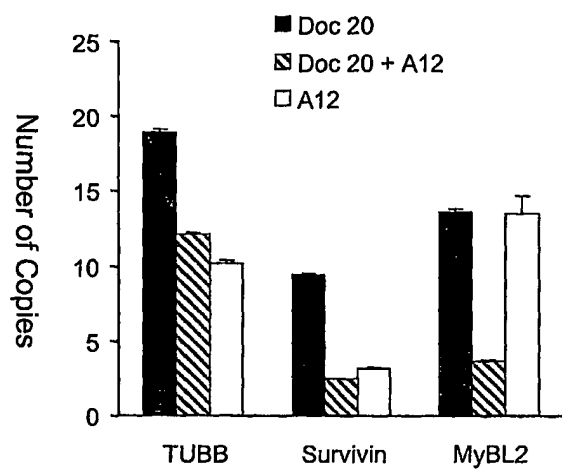


Fig. 4

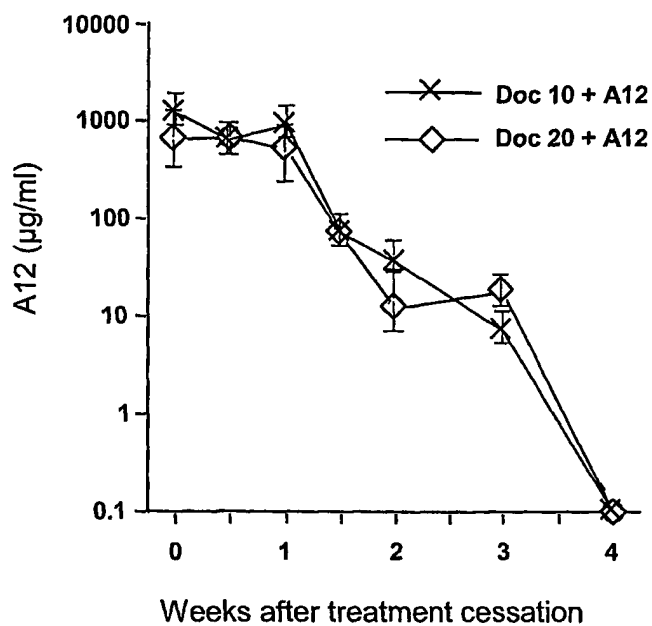


Fig. 5

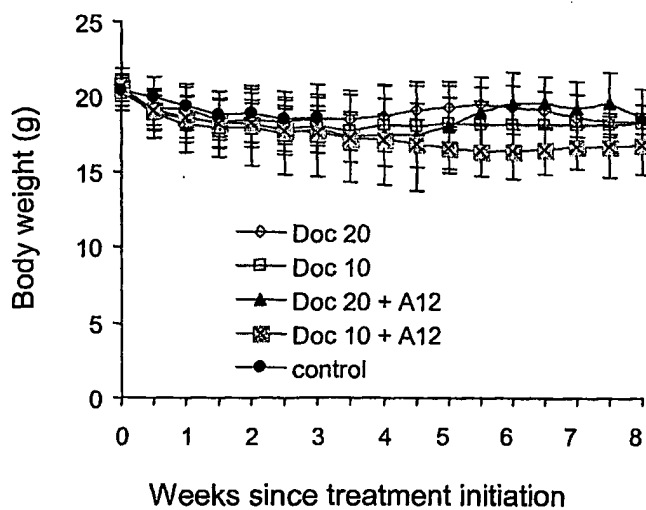


Fig. 6

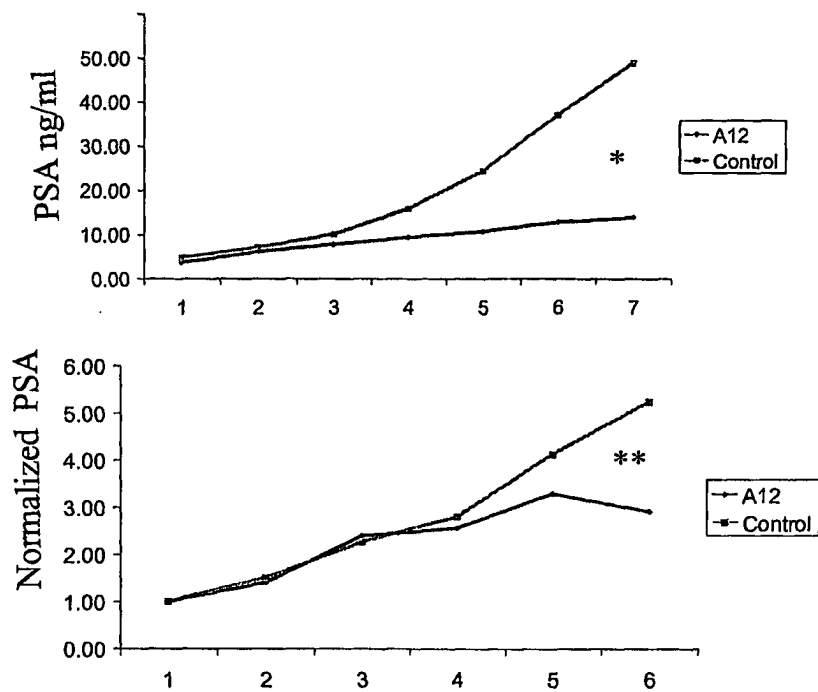


Fig. 7

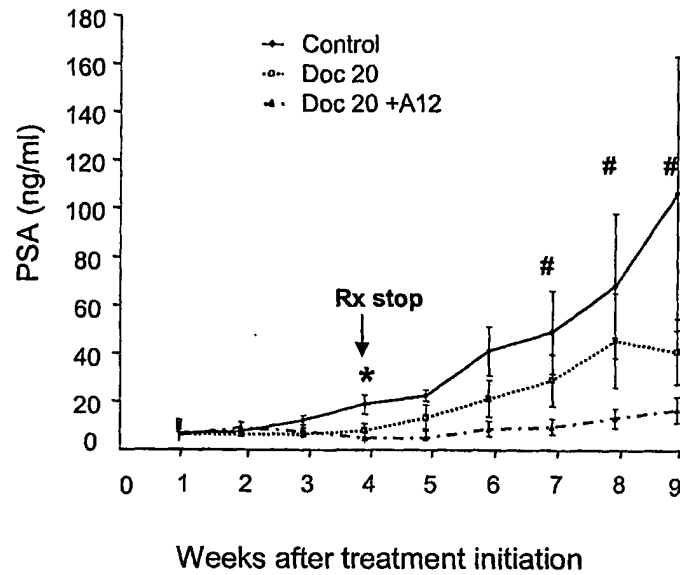


Control



A12

Fig. 8

a.**b.**



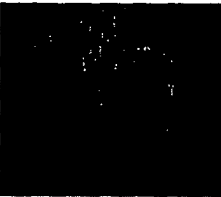
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PSA (ng/ml)	87	30	0
BMD (units)	0.19	0.17	0.06

Fig. 9

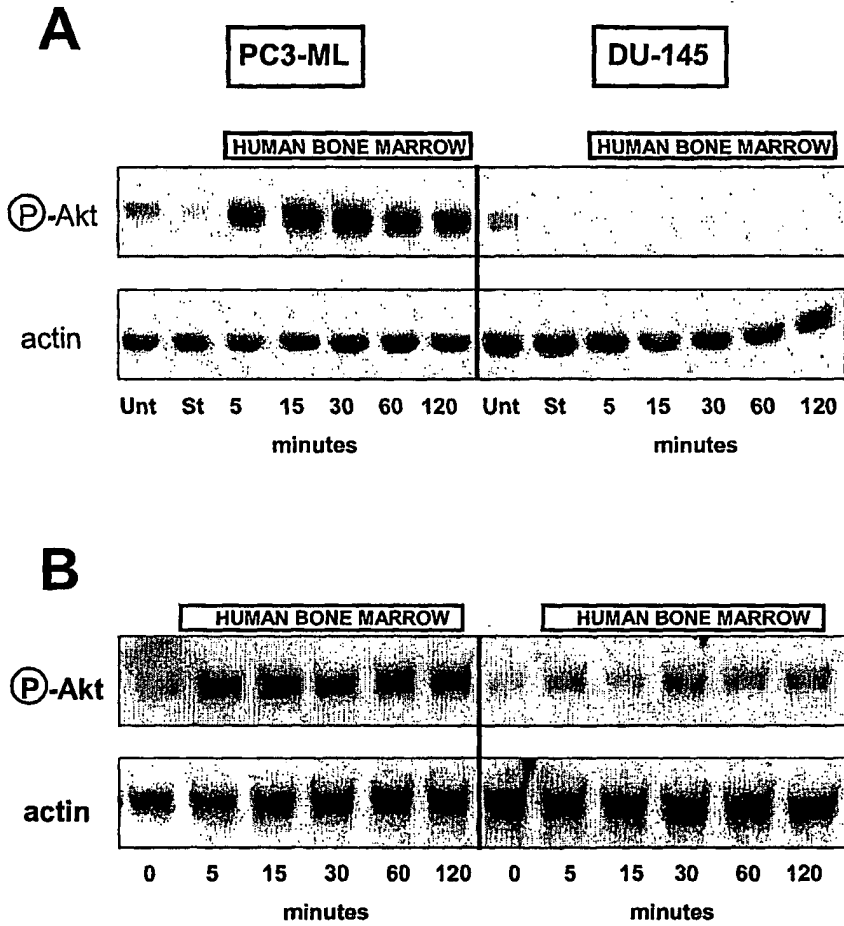


Fig. 10

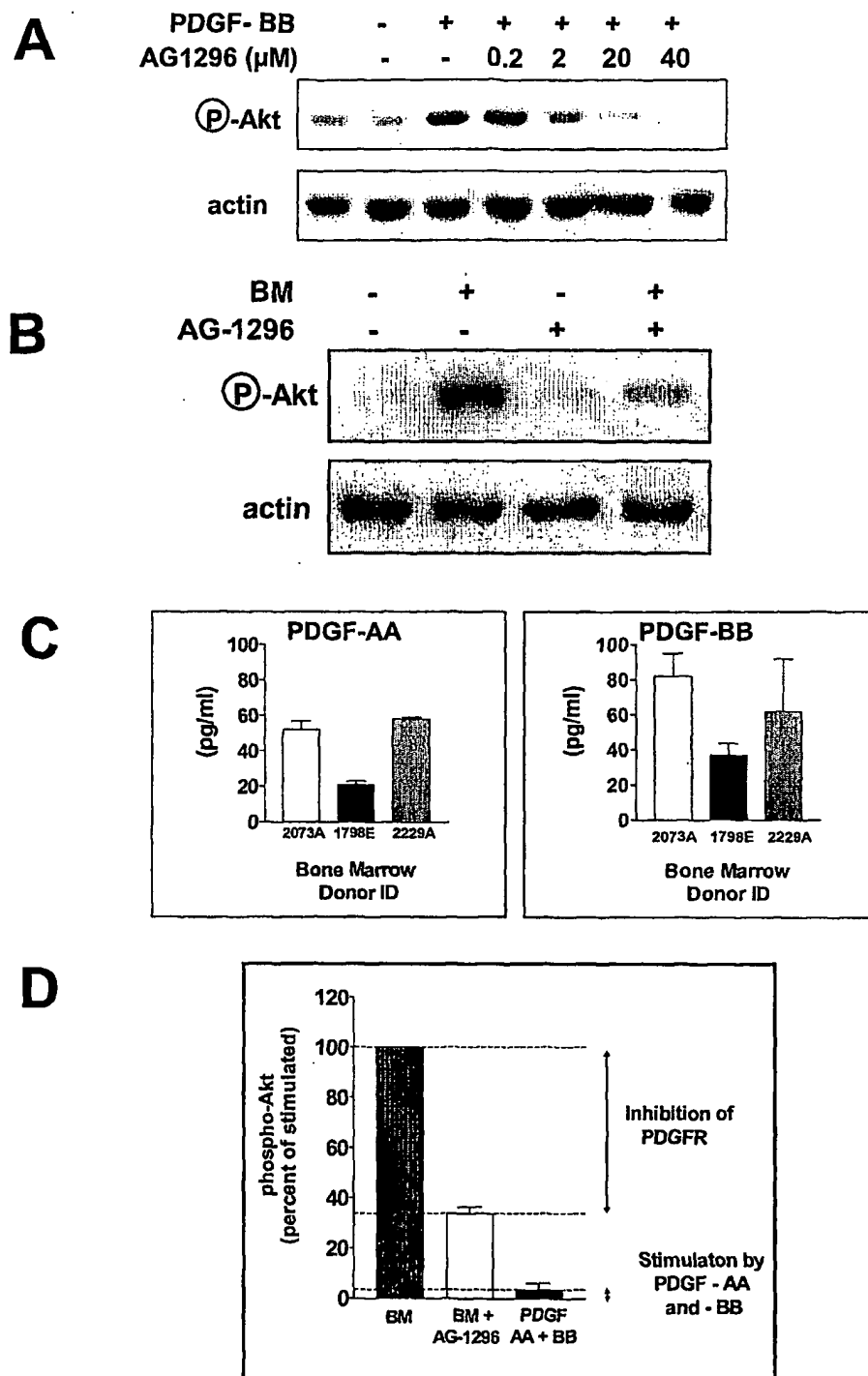


Fig. 11

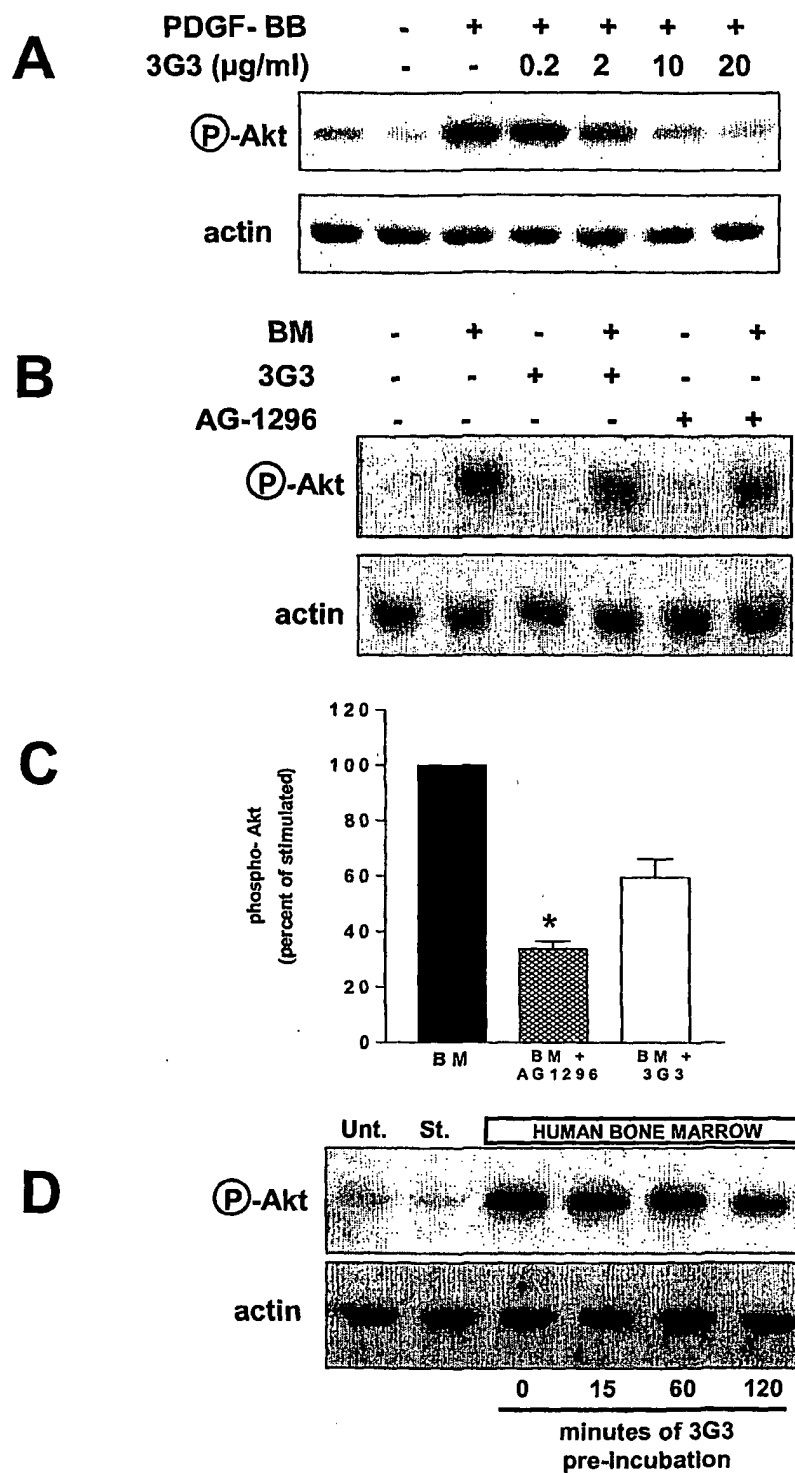


Fig. 12

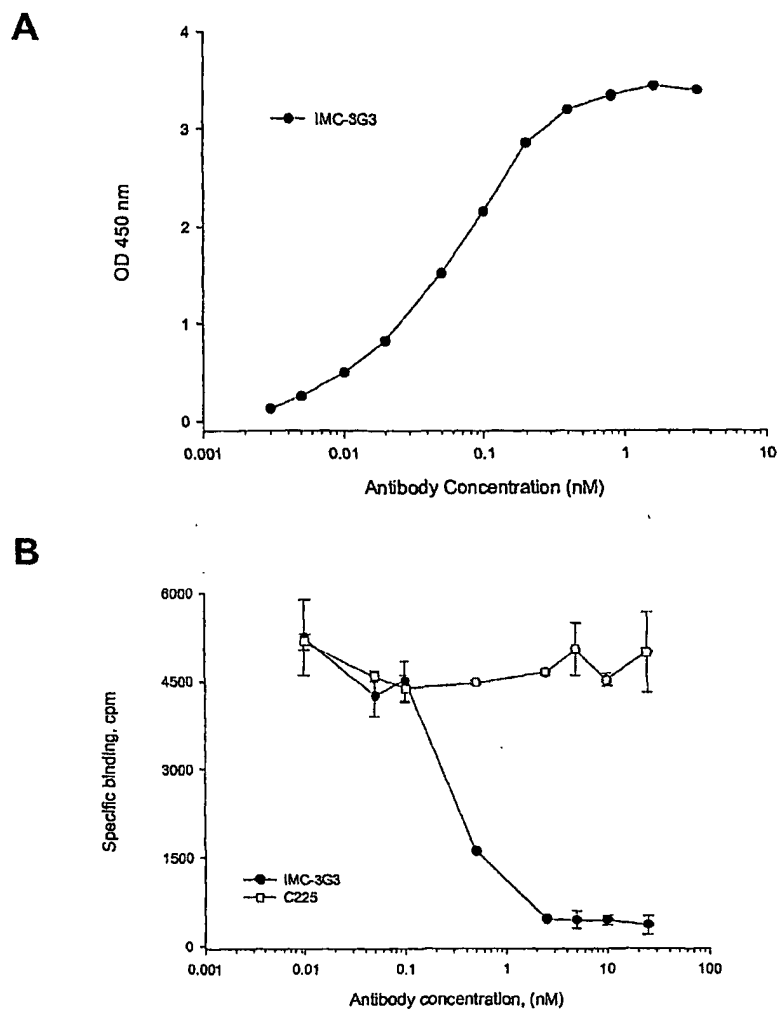


Fig. 13

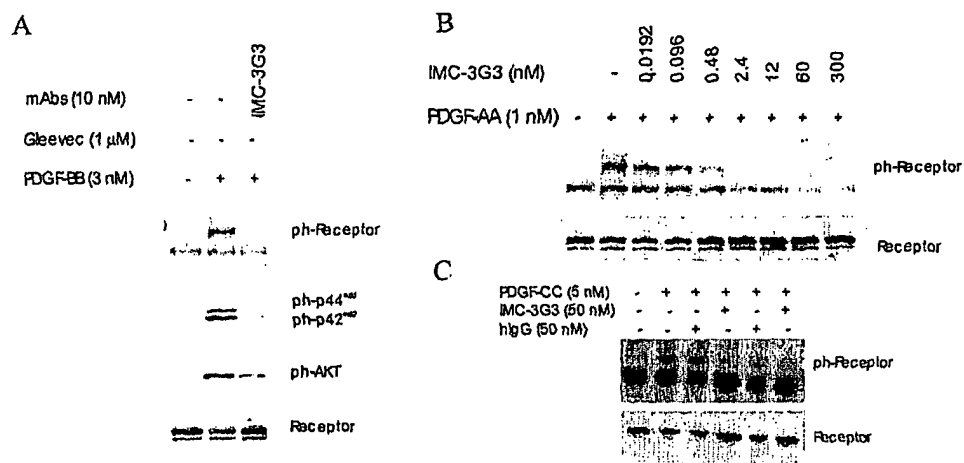


Fig. 14

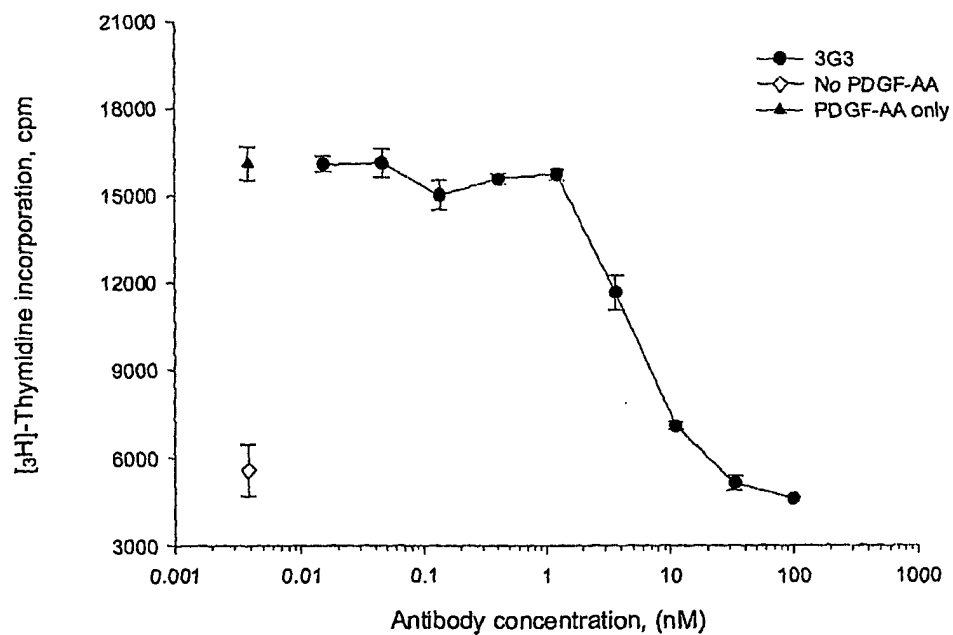


Fig. 15

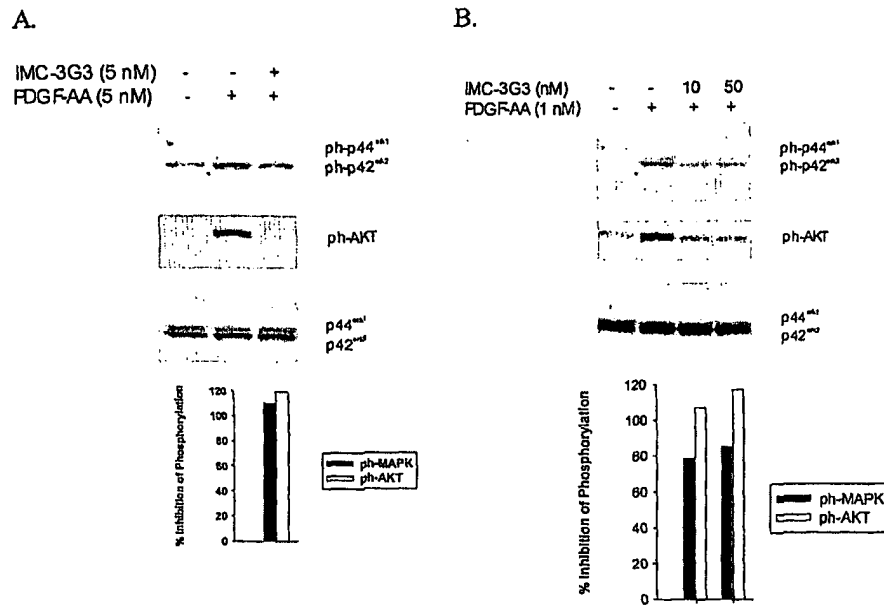


Fig. 16

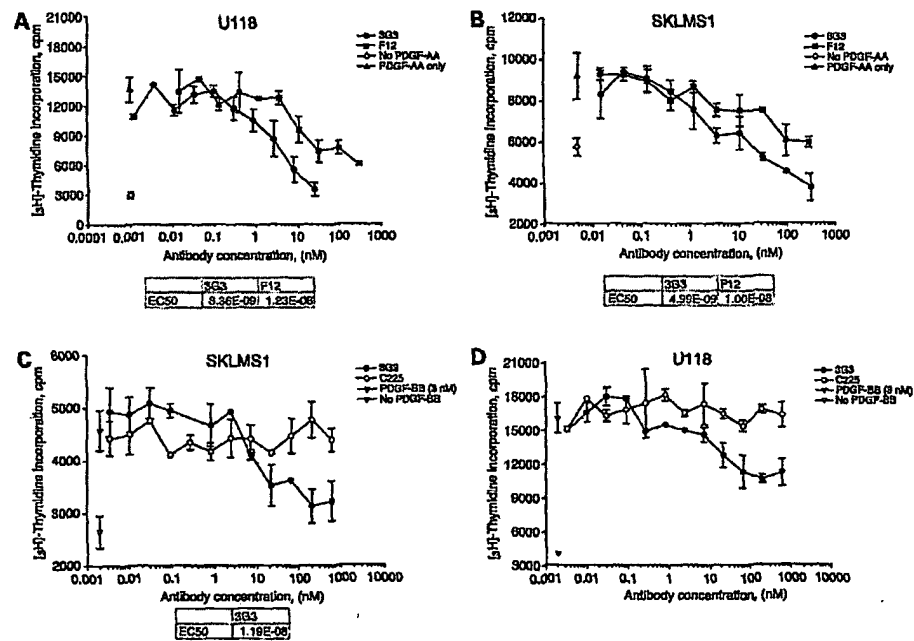


Fig. 17

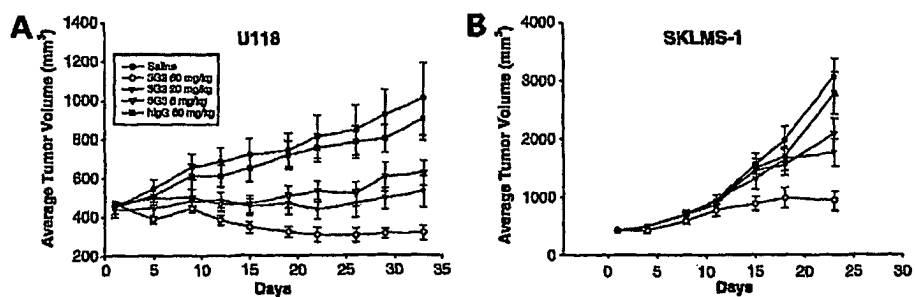


Fig. 18

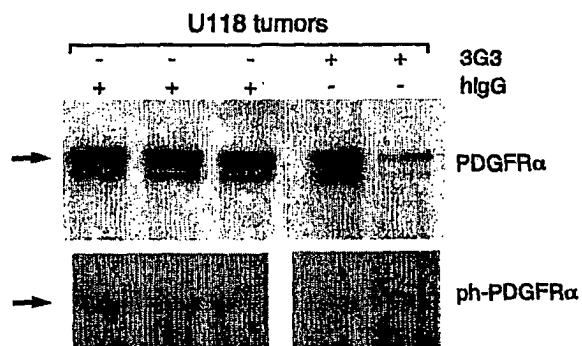


Fig. 19

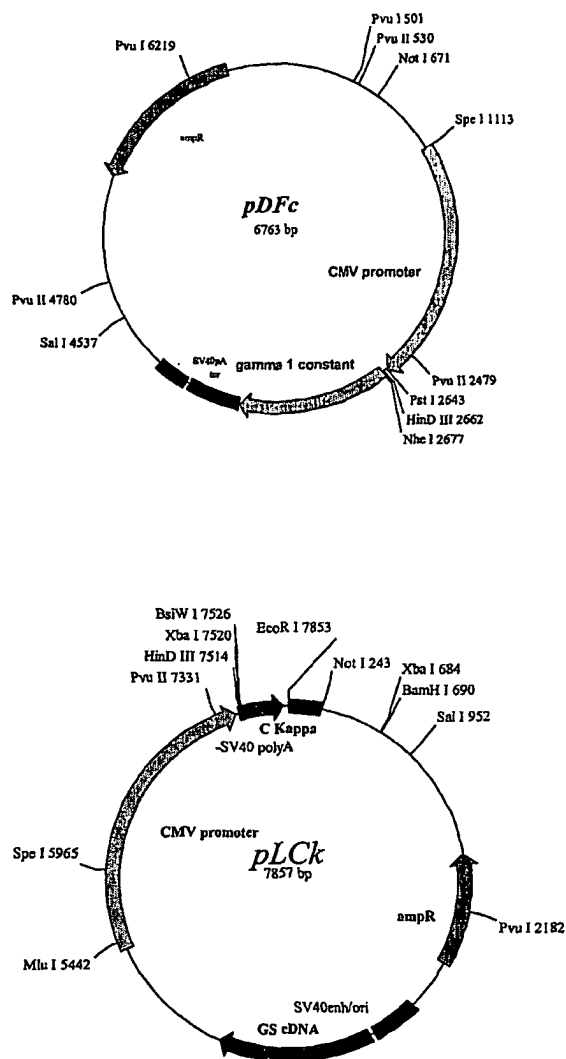


Fig. 20